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(54) Title: COMPOSITIONS AND METHODS FOR INHIBITION OF CANCER INVASION AND ANGIOGENESIS

(57) Abstract: The invention provides antibodies that specifically bind a membrane protease complex, the complex consisting of two homodimers of seprase and dipeptidyl peptidase IV (DPPIV), obtained from mammalian, preferably human cell membranes. The antibodies specifically bind the DPPIV protease of the seprase-DPPIV complex. This membrane protease complex resides on cell surface invadopodia at the leading edge of angiogenic endothelia, migratory fibroblasts, and invading cancer cells. The antibodies and immunoconjugates of the invention specifically bind the membrane protease complex at the cell surface invadopodia, yet fail to react with resting cells in adjacent human tissues and blood vessels. These antibodies and immunoconjugates block interaction of collagen matrix with the seprase-DPPIV complex in the invasive cells during angiogenesis and cancer spreading but not that with other endothelia or tumor cells. The invention further provides methods for identifying and of using DPPIV antagonists to inhibit capillary sprouting, angiogenesis and cancer invasion in tumor tissues and metastases. Also provided are therapeutic compositions comprising DPPIV antagonists.

COMPOSITIONS AND METHODS FOR INHIBITION OF CANCER INVASION AND ANGIOGENESIS

This work was supported by grants from one or more of the following: U.S. Public Health Service, National Cancer Institute and The National Institute of Aging. The government may have certain rights to this invention.

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Serial No. 09/541,785 which was filed on April 3, 2000, which claims the benefit of provisional application U.S. Serial No. 06/193,987 filed on April 1, 2000.

FIELD OF THE INVENTION

The present invention relates generally to the field of medicine, and relates specifically to angiogenesis and metastasis of cancer tissues, as well as treatment of cardiovascular disease. Specifically the invention relates to the use of modulators, including antagonists and agonists of the serine integral membrane protease, dipeptidyl peptidase IV (DPPIV also known as CD26).

BACKGROUND OF THE INVENTION

Growth of new blood vessels (angiogenesis) plays a key role in tissue repair and in cancer progression. The invasion of cells into a connective tissue barrier during angiogenesis requires remodeling of the extracellular matrix (ECM) by migratory cells (Martin, 1997). In cancer invasion such cellular activities occur on membrane protrusions invadopodia (Chen,1979) which exhibit dynamic membrane mobility, ECM adhesion and degradation. Thus, cellular invasion is an important process for cancer metastasis (Stetler-Stevenson et al., 1993). Several classes of proteases including matrix metalloproteinases (MMPs), serine proteases, cysteine proteases (cathepsin B and cathepsin L), and aspartic acid proteases (cathepsin D) can degrade proteins in the ECM (Chen, 1992). And invading cancer cells possess ECM degrading proteolytic enzymes that are concentrated at specialized plasma membrane

protrusions, termed invadopodia (Chen et al., 1994). Recent studies showed that integral membrane proteases might contribute significantly to ECM degradation and ultimately cancer invasion by virtue of their location at invadopodia (Monsky and Chen, 1993).

Recent evidence has demonstrated the involvement of serine-integral membrane proteases (SIMP), including dipeptidyl peptidase IV (DPPIV)/CD26 and seprase, in cell surface proteolysis (Chen, 1996). SIMP members are type II transmembrane proteins, with cytoplasmic tails that contain 6 amino acids (a.a.) followed by a transmembrane domain of 20 a.a. (in the case of seprase) or 22 a.a. (in the case of DPPIV) at the N-terminus and a stretch of 200 a.a. at the C-terminus that constitutes a catalytic region with the catalytic serine in a non-classical orientation (Goldstein et al., 1997; Pineiro-Sanchez et al., 1997).

DPPIV specifically removes N-terminal dipeptides from oligo-peptides, which include Neuro-Peptide Y and other peptide hormones, with either L-proline, L-hydroxyproline, or L-alanine at the penultimate position (Heins et al., 1988, Walter et al., 1980). DPPIV has been shown to be an adhesion receptor for collagen (Bauvois, 1988; Hanski et al., 1988; Loster et al., 1995) or fibronectin (Cheng et al., 1998; Johnson, et al., 1993; Piazza et al., 1989). In addition, a recent report showed that DPPIV also possesses a seprase-like gelatinase activity and therefore endopeptidase activity (Bermpohl et al., 1998), suggesting its involvement in collagen degradation. DPPIV is expressed constitutively on brush border membranes of intestine and kidney epithelial cells (Yaron and Naider, 1993; Morimoto and Schlossman, 1994).

Seprase, originally identified as a 170 kDa membrane-bound gelatinase is expressed on invadopodia of highly aggressive melanoma LOX cells (Aoyama and Chen, 1990; Mueller et al., 1999; Monsky et al., 1994). The active enzyme is a homodimer of 97 kDa subunits, which are proteolytically inactive (Pineiro-Sanchez et al., 1997). Analysis of the deduced amino acid sequence from a cDNA that encodes the 97 kDa subunit (Goldstein et al., 1997) revealed that it is homologous to DPPIV, and is essentially identical to fibroblast activation protein α (FAPα) (Scanlan et al., 1994), which is expressed on reactive stromal fibroblasts of epithelial cancers and healing wounds (Garin-Chesa et al., 1990). In addition, DNA and protein analysis of

embryonic tissues has suggested potential additional members of SIMP (Bermpohl et al., 1998).

A growing body of evidence indicates that angiogenesis is essential to the progression of cancer. Angiogenesis is the sprouting of new capillaries from preexisting blood vessels. Normally, angiogenesis in mammals is confined to the reproductive system, embryogenesis and development, and repair after injury. However, angiogenesis can also occur in pathological conditions such as cancer, retinal neovascularization, neovascularization in atherosclerotic plaques, hemangiomas, arthritis, and psoriasis. See Folkman, 1995. Without vascularization, tumors may remain for years as small (less than a few millimeters) asymptomatic lesions. Weidner et al. (1991). Angiogenesis allows the cancer cells access to the circulatory system. The new blood vessels also provide a gateway for cancer cells to enter the circulation and metastasize to distant sites (Folkman 1990; Klagsbrunn and Soker, 1993).

As in cancer cell invasion, angiogenesis involves matrix degradation by migrating endothelial cells at the invasion front; proteases including matrix metalloproteases (MMPs) (Hiraoka et al., 1998; Brooks et al., 1998) and plasminogen activators (Pepper et al., 1993) are essential. However, novel membrane-bound proteases active at sites of angiogenesis have not yet been identified.

Several approaches for inhibition of angiogenesis have been proposed as useful therapies for restricting tumor growth. These include inhibition of angiogenesis by (1) inhibition of release of "angiogenic molecules" such as VEGF (Vascular endothelial growth factor) and basic.FGF (fibroblast growth factor), (2) neutralization of angiogenic molecules, such as by use of anti-b.FGF antibodies, (3) inhibition targeted to $alpha_v.beta_3$ integrin, and (4) inhibition of the endothelial cell response to angiogenic stimuli. This latter strategy has received particular attention, and Folkman et al., Cancer Biology, 3:89-96 (1992), have described several endothelial cell response inhibitors, including collagenase inhibitor, basement membrane turnover inhibitors, angiostatic steroids, fungal-derived angiogenesis inhibitors, platelet factor 4, thrombospondin, arthritis drugs such as D-penicillamine

and gold thiomalate, vitamin D_3 analogs, alpha-interferon, and the like that might be used to inhibit angiogenesis.

Monoclonal antibodies (MAbs) to human tumor-associated differentiation antigens offer promise for the "targeting" of various antitumor agents such as radioisotopes, chemotherapeutic drugs, and toxins. [Order, in "Monoclonal Antibodies for Cancer Detection and Therapy", Baldwin and Byers, (eds.),London, Academic Press (1985)]. In addition, some monoclonal antibodies have the advantage of killing tumor cells via antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) in the presence of human effector cells or serum [Hellstrom et al., Proc. Natl. Acad. Sci. USA 83:7059-7063 (1986)], and there are a few monoclonal antibodies that have a direct antitumor activity which does not depend on any host component [Drebin et al., Oncogene 2:387-394 (1988)].

For additional proposed inhibitors of angiogenesis, see Blood et al., Bioch. Biophys. Acta., 1032:89-118 (1990) for a general review of angiogenesis and tumor metastasis; also Moses et al., Science, 248:1408-1410 (1990) describes a protein inhibitor of angiogenesis derived from cartilage; and Ingber & Folkman, Lab. Invest. 59:44-51 (1988) describes inhibition of angiogenesis through modulation of collagen metabolism.

U.S. Pat. No. 5,092,885, of Yamada et al. discloses laminin peptides with angiogenesis-blocking activity. U.S. Pat. No. 5,112,946 of Maione et al. discloses modified PF4 compositions as inhibitors of angiogenesis. U.S. Pat. No. 5,192,744, discloses human thrombospondin for use as an inhibitor of angiogenesis. U.S. Pat. No. 5,202,352 discloses intravascular embolizing agents containing angiogenesis inhibiting substances in oils, emulsions or suspensions. U.S. Pat. No. 5,766,591 discloses antagonists of vitronectin $\alpha_v.\beta_3$ as angiogenesis inhibitors.

U.S. Pat. No. 5,980,896 of Hellstrom et al. discloses antibodies and immunoconjugates reactive with human carcinomas and is especially useful in practicing the full scope of the present invention. Among the disclosed compositions and methods which are especially applicable to the present invention are: chimeric antibodies, immunoconjugates thereof and their methods of preparation and use; and anti-tumor drugs, cytotoxins, radioactive agents and enzymes useful in

immunoconjugate compositions. The specification of U.S. Pat. No. 5,980,896 is hereby incorporated by reference in its entirety.

There is still a need, however for novel and more effective angiogenesis modulation therapies for use alone or in combination with one or more of the currently available therapies for treatment of growth and proliferative disorders involving angiogenesis.

SUMMARY OF THE INVENTION

The invention provides monospecific antibodies that specifically bind an epitope of a mammalian DPPIV (dipeptidyl peptidase IV/CD26), such as a human DPPIV. Preferably, the monospecific antibodies inhibit angiogenesis and tumor invasiveness.

Also provided are bispecific antibodies with binding specificity for two epitopes, one being an epitope of DPPIV. Bispecific antibodies of the present invention include those in which the second epitope bound is an epitope of seprase, MT1-MMP, MMP-2 or α(3)β(1)-integrin.

The present invention further provides immunoconjugates comprising a monospecific or a bispecific antibody which specifically binds an epitope of human DPPIV and inhibits angiogenesis, the antibody being joined to a therapeutic agent.

In yet another aspect the invention provides pharmaceutical compositions for inhibiting angiogenesis comprising an effective amount of an antibody which specifically binds an epitope of a mammalian DPPIV and inhibits angiogenesis, the antibody being formulated in a pharmaceutically acceptable carrier.

In yet another aspect the invention provides pharmaceutical compositions for inhibiting angiogenesis comprising an effective amount of an immunoconjugate of an antibody which specifically binds an epitope of a mammalian DPPIV and inhibits angiogenesis, the antibody being formulated in a pharmaceutically acceptable carrier.

The present invention yet further provides a method of treating a mammal suffering from a growth or proliferative disorder associated with angiogenesis, comprising administering to the site of angiogenesis an effective amount of an

antibody which specifically binds an epitope of a human DPPIV and inhibits angiogenesis.

In yet another aspect the present invention provides a method of treating a mammal suffering from a growth or proliferative disorder involving angiogenesis, comprising administering to the site of angiogenesis an effective amount of an immunoconjugate which specifically binds an epitope of a human DPPIV and inhibits angiogenesis.

In a further aspect the present invention provides continuous cell lines which produce monospecific antibodies that specifically bind an epitope of a mammalian DPPIV and thereby inhibit angiogenesis.

In a yet further aspect the present invention provides a method of stimulating angiogenesis in a mammal suffering from disease or disorder that may be remedied by an increased blood supply, such as for instance cardiovascular disease, by administering an angiogenesis-stimulating amount of a DPPIV stimulator, whereby the blood supply to the affected tissue is increased.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Seprase and DPPIV expression in the endothelial cells of sprouting vessels but not in differentiated vessels in human malignant breast carcinoma. Both seprase (middle panel) and DPPIV (upper panel) are present in the endothelial cells of sprouting microvessels (brown stains indicated by black solid arrows) but not in endothelia of adjacent vessels (open arrows). Adjacent to microvessels are cell clusters of invasive breast carcinoma that stain positively for seprase and DPPIV. The bottom panel is an expanded view of seprase staining of vessels. Paraffin sections of breast carcinoma tissue were stained with the anti-seprase mAb D28 or the anti-DPPIV mAb E26. Bar = 100 μm.

Figure 2. Enhanced expression and proteolytic activities of seprase and DPPIV in migratory endothelial cells. **a-e,** Seprase/DPPIV expression, proteolytic activities and mRNA profiles were analyzed in confluent (+) and sparse (-) HUVEC. **a,** Immunoblotting analysis of cell lysates using anti-seprase (D28, Piniero-Sanchez et al., 1997), DPPIV (E26), β1-integrin (C27, Bloch et al., 19977) and antibody control.

The immunoblots and their densitometry scans (adjoining panels) show that both seprase and DPPIV are elevated in sparse cultures, while β1-integrin remains the same in confluent (+) and sparse (-) conditions. **b,** Gelatin zymography of cell lysates in the presence of Ca++ (+2 mM $CaCl_2$) and deprived of Ca++ (+ 2 mM EDTA). The 170-kDa gelatinase (seprase) activity was elevated in sparse cultures, while the 62-kDa MMP-2 activity remained the same in confluent (+) and sparse (-) conditions. **c,** DPPIV substrate Gly-Pro-AFC (7-Amino-4-Trifluoromethyl Couramin) overlay of cell lysates. The 200-kDa DPPIV activity was increased in the sparse culture. **d,** Detection of seprase RNA. RT-PCR was carried out on total RNA from LOX human malignant melanoma cells (Lox) - a positive control (Goldstein et al., 1997) and confluent (+) and sparse (-) HUVEC using oligonucleotide primers "FAP1 +2" and "FAP11 +4" that correspond to specific nucleotide positions of the seprase cDNA as described (Goldstein et al., 1997). Minus reverse transcriptase controls are shown in lanes marked "RT". Similar amounts of glyceraldehyde-3-phosphate-dehydrogenase mRNA were detected in both the confluent and sparse HUVEC (Lanes marked GPDH). These results suggest that the increased expression of seprase detected in sparse HUVEC is not due to an upregulation in the seprase mRNA level; but instead is due to increased translational efficiency of the seprase mRNA and/or increased stability of seprase itself. **e,** Detection of DPPIV RNA. RT-PCR was carried out on total RNA from confluent (+) and sparse (-) HUVEC using oligonucleotide primers DPPF1+DPPR2 that correspond to nucleotide positions #24 - 43 (5' UTR) and #2798 - 2781 (3' UTR) in human DPPIV cDNA. **f-g,** Immunofluorescence distribution of seprase and DPPIV in migratory HUVEC stimulated by wounding (indicated by arrows) of the monolayer (central panels). The wound monolayer was stained three hours later with antibodies against DPPIV (E26) and β1 (C27) or seprase (D28) and β1 (C27), respectively. Bar = 10 μm. **h,** Morphology of HUVEC migration at time 0 and 24 hours after wounding of the monolayer (panels marked 24 hr). The wound was closed within 24 hours but cell migration could be blocked by mAbs E19 or E26 against DPPIV. **i,** Dose-dependent inhibition of cell migration by inhibitory mAb E19 (against DPPIV; - □ open squares) and C27 (β1; open triangles) but not by control mAb E3 against DPPIV (DPPIV solid triangles) or C37 (against cell surface

glycoprotein gp90; solid circles). Three experiments of 4 h monolayer wound models were carried out for each antibody. Cell migration was quantified by measuring the areas of cell advancement from the original wound edge. The values are mean ± SD. **j,** Time-course of antibody inhibition of cell migration. All antibodies, mAb E19 (against DPPIV; - □ open squares), C27 (β1; open triangles), E3 (DPPIV solid triangles), C37 (glycoprotein gp90; solid circles), or buffer alone (Control; solid diamonds) were applied at 5 μg per ml. Experimental conditions were the same as panel i above.

Figure 3. Inhibition of endothelial tube formation by a mAb to DPPIV. **a,** Morphology of HUVEC tube formation in Matrigel assay (Control). It could be blocked by mAb E19 or E26 (DPPIV). Bar = 100 μm. **b,** Inhibition of endothelial tube formation. All antibodies, mAb E26 (against DPPIV), C27 (β1), E3 or C37 (control IgG), or buffer alone (Control) were applied at 5 μg per ml prior to tube formation when cells adhered to Matrigel. The matrix metalloprotease inhibitor CT1847 was added at 10 nM in the presence of 0.01% DMSO and 0.01% DMSO was used as vehicle control (+DMSO). Three experiments for each antibody or inhibitor were used in this plot. Tube formation was quantified by measuring the areas of tubes in each well. The values are mean ± SD. **c,** Inhibition of preexisting endothelial tubes by antibodies to DPPIV and β1 or the matrix metalloprotease inhibitor CT1847. Experimental conditions were identical to above except antibodies and inhibitors were applied after tubes were formed. **d,** Immunofluorescent distribution of seprase in migratory HUVEC (indicated by arrow) from a tube in Matrigel. The HUVEC culture (phase contrast image shown in the left panel) was stained with antibodies against seprase (D8) (right panel), respectively. Bar = 10 μm.

Figure 4. Inhibition of human angiogenesis *in vitro* by a mAb to DPPIV. **a,** Morphology of VEGF- and bFGF-induced HDMEC capillary sprouts and capillary networks (VEGF/bFGF – and +), which could be blocked by mAb E19 or E26 (+ DPPIV). Bar = 200 μm. **b,** Inhibition of HDMEC capillary sprouts. All antibodies, mAb E26 (+ DPPIV), mAb C27 (+ β1), and E3 or C37 (+ control IgG), were applied at 20 μg per ml to impregnated fibrin gels. The matrix metalloprotease inhibitor

CT1847 was added at 40 nM in the presence of 0.01% DMSO; 0.01% DMSO was used as vehicle control. Three experiments for each antibody or inhibitor were performed in this plot. Capillary sprouting was quantified by measuring the number of tubes and beads in each well. The ratio of tubes/beads occurring with VEGF/bFGF+ samples was arbitrarily set at 100%. The values are mean ± SD.

Figure 5. Experimental procedure for DPPIV/Seprase antigen capture and inhibition assay. Plates were coated with rabbit anti-rat IgG (1:4000 dilution) in blocking buffer (5% fat free milk in 1xPBS)and washed (in washing buffer: 0.05% Tween-20 in 1xPBS). The wells were then incubated with the capture antibody (E3-anti-DPPIV, D8-anti-seprase, D28-anti-seprase, or normal rat serum = supernatant). Alternatively, wells were then incubated with an inhibitory anti-DPPIV antibody E19, or E26 as capture antibody. Wells were washed 5X and incubated with second antibody and again washed 5X. Cell lysates (HS578T, MDA436, WI38 or LOX) or lysates pretreated with anti-DPPIV antibody E19 or E26 were incubated in coated wells, washed and assayed for DPPIV peptidase activity. Antibody reactions were carried out at 37°C for 4hrs. DPPIV peptidase assays were developed with chromogenic substrate Gly-Pro-pNA (2.15mM) or fluorogenic substrate Gly-Pro-AMC (14.6mM).

Figure 6: HS578T and LOX cell lysate DPPIV activity inhibition by mAb E19 or mAb E26. DPPIV activity from cell lysates HS578T (panels A and B) or LOX (panels C and D) measured after antibody binding: with E19 (panels A and C) or E26 (panels B and D). Control: (lightly shaded columns), the 96well plate was first coated with rabbit anti-rat IgG, washed 5X and then coated with the first mAb. Lysates were then bound. Direct inhibition: (black columns) the 96well plate was first coated with rabbit anti-rat IgG, washed 5X and then coated directly with E19 or E26. Lysates were then bound. Indirect inhibition (medium shaded columns) the 96well plate was first coated with rabbit anti-rat IgG, washed 5X and then coated with the anti-seprase/DPPIV mAb. Lysates were then bound. After washing, E19 or E26 was added and assayed for peptidase activity.

Figure 7. The detection of mRNA and protein expression of seprase and DPPIV in MDA-MB-436, MDA-MB-231, and MCF7 breast cancer cells by RT-PCR

and Western immunoblotting analyses. A and B, RT-PCR was carried out on MDA-MB-231, MDA-MB-436 and MCF7 RNAs using DPPIV and seprase specific primers, respectively. Only MDA-MB-436 cells could be shown to produce the seprase PCR product of ~2.4 kb, which encodes 97-kDa subunit (A). Human WI-38 fibroblast RNA, used as a positive control, generated an identical amplicon pattern as the MDA-MB-436 breast carcinoma cells. A DPPIV PCR product of ~2.8 kb, which encodes the 110-kDa subunit, was detected in the positive control WI-38, andfrom MDA-MB-436 and MCF7 RNAs on agarose gels containing ethidium bromide (B). The two intense lower bands detected in the MCF7 lane were also present in the minus reverse transcriptase control (data not shown). C-E, Western immunoblotting analysis of seprase and DDPIV expression in breast carcinoma cells using the mAbs: D8 and E97 directed against seprase and E19 against DPPIV. The mAb D8 recognizes both seprase and the 97-kDa subunit (C), but mAb E97 recognizes only the monomeric 97-kDa subunit (D) and reveals better total seprase in the cell lysate. Similar to RT-PCR analysis, only MDA-MB-436 cells expressed seprase (C and D), while both MDA-MB-436 and MCF7 breast carcinoma cells expressed DDPIV (E). The mAb D8 recognized the 170-kDa seprase and its 97-kDa subunit in the MDA-MB-436 breast carcinoma cells under nonreduced and nonboiled conditions (F, lane 1), whereas only 97-kDa protein was observed under reduced and boiled conditions (F, lane 2).

Figure 8. Protein expression and inhibition of collagen degradation of MDA-MB-436 cells transfected with seprase sense (pA15), seprase anti-sense (pA26), plasmid (pA11), and DPPIV ribozyme (Rbz8) constructs. Cell lysates of stable transfectants of the MDA-MB-436 parent line, which has integrated various construct expression vector (pCR3.1) were analyzed by Western blotting using antibodies against seprase and DPPIV. Rbz 8 blocks DPPIV protein expression in MDA-MB-436 cells. Cells transfected with antisense seprase (p26) or DPPIV ribozyme (Rbz8) show a partial inhibition of collagen-degrading activities. TRITC collagen peptide release is shown as ng per ml medium.

Fig. 9. Collagen-degrading activity of the seprase-DPPIV complex. Collagen-degrading activities of the protease complex and purified DPPIV were determined by the release of peptide fragments from biotinylated type I collagen gel

in the presence of these enzymes. The seprase-DPPIV complex was isolated from WI38 RIPA extracts using mAbs D28 (lane marked Ip Seprase) and E3 (lane marked Ip DPPIV) beads, seprase from LOX WGA fraction using mAb D28 (lane marked Ip Seprase*), MMP-2 from media conditioned by MMP-2 COS cell transfectants (lane marked MMP-2), and proteins associated with β3 integrin were from WI38 RIPA extracts using HB242 (lane marked Ip β3 integrin). All enzymes, including the protease complex, purified seprase, MMP-2, and proteins associated with β1 or β3 integrin were applied at the concentration of 50 ng/ml. Biotinylated type I collagen and enzymes were incubated for 8 h at 37°C with proteins described above, plus the mAb C37 control (5 μg/ml, lane marked Control) or inhibitory anti-DPPIV mAb E26 (5 μg/ml, lanes marked +E26), the serine-proteinase inhibitor AEBSF (20 μM, lanes marked +AEBSF), and the metallo-proteinase inhibitor CG1847 (50 nM, lanes marked + CG1847). Profiles of collagen peptide release (shown in panels at right of the figure) were scanned with a densitometer. The seprase-DPPIV complex degrades collagen fibers to produce a novel multiple-peptide pattern.

DETAILED DESCRIPTION OF THE INVENTION

Definitions: As used herein, the following words or phrases have the specified meanings.

As used herein, "fragment" is defined as at least a portion of the variable region of the immunoglobulin molecule which binds to its target, i.e. the antigen binding region. Some of the constant region of the immunoglobulin may be included.

As used herein, an "immunoconjugate" means any molecule or ligand such as an antibody or growth factor chemically or biologically linked to a cytotoxin, a radioactive agent, an anti-tumor drug or a therapeutic agent. The antibody or growth factor may be linked to the cytotoxin, radioactive agent, anti-tumor drug or therapeutic agent at any location along the molecule so long as it is able to bind its target. Examples of immunoconjugates include immunotoxins and antibody conjugates.

As used herein, "selectively killing" means killing those cells to which the antibody binds. As used herein, examples of "carcinomas" include, but are not limited to the following: bladder, breast, colon, liver, lung, ovarian, and pancreatic carcinomas. As used herein, "immunotoxin" means an antibody or growth factor chemically or biologically linked to a cytotoxin or cytotoxic agent.

As used herein, an "effective amount" is an amount of the antibody, immunoconjugate, recombinant molecule which kills cells or inhibits the proliferation thereof. As used herein, "competitively inhibits" means being capable of binding to the same target as another molecule. With regard to an antibody, competitively inhibits mean that the antibody is capable of recognizing and binding the same antigen binding region to which another antibody is directed.

As used herein, "antigen-binding region" means that part of the antibody, recombinant molecule, the fusion protein, or the immunoconjugate of the invention which recognizes the target or portions thereof. As used herein, "therapeutic agent" means any agent useful for therapy including anti-tumor drugs, cytotoxins, cytotoxin agents, and radioactive agents.

As used herein, "anti-tumor drug" means any agent useful to combat cancer including, but not limited to, cytotoxins and agents such as antimetabolites, alkylating agents, anthracyclines, antibiotics, antimitotic agents, procarbazine, hydroxyurea, asparaginase, corticosteroids, mytotane (O,P'-(DDD)), interferons and radioactive agents. As used herein, "a cytotoxin or cytotoxic agent" means any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof.

As used herein, a chemotherapeutic regimen refers to any treatment with a chemotherapeutic agent. Examples of chemotherapeutic agents include, for example, the anti-tumor drugs listed above.

As used herein, "a radioactive agent" includes any radioisotope which is effective in destroying a tumor. Examples include, but are not limited to, cobalt-60 and X-ray emitters. Additionally, naturally occurring radioactive elements such as uranium, radium, and thorium which typically represent mixtures of radioisotopes, are suitable examples of a radioactive agent.

As used herein, "administering" means oral administration, administration as a suppository, topical contact, intravenous, intraperitoneal, intramuscular or subcutaneous administration, or the implantation of a slow-release device such as a miniosmotic pump, to the subject. As used herein, "curing" means to provide substantially complete tumor regression so that the tumor is not palpable.

As used herein, "tumor associated antigens" means any cell surface antigen which is generally associated with tumor cells, i.e., occurring to a greater extent as compared with normal cells. Such antigens may be tumor specific. Alternatively, such antigens may be found on the cell surface of both tumorigenic and non-tumorigenic cells. These antigens need not be tumor specific. However, they are generally more frequently associated with tumor cells than they are associated with normal cells.

As used herein, "pharmaceutically acceptable carrier" includes any material which when combined with the antibody retains the immunogenicity of the antibody and is non-reactive with the immune systems of the subject. Examples include, but

are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets including coated tablets and capsules.

Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods.

The invention

The invention provides monospecific antibodies which specifically bind an epitope of a human DPPIV (dipeptidyl peptidase IV/CD26) and inhibits angiogenesis. The monospecific antibodies of the present invention include monoclonal, chimeric and humanized antibodies and antibody fragments that specifically bind the epitopes bound by either of the anti-DPPIV antibodies E19 or E26. In a preferred embodiment the antibody fragments comprise the antigen-binding region of the antibody.

In one embodiment the monospecific antibodies of the present invention include those that bind specifically with the catalytic or substrate-binding domains of human DPPIV (dipeptidyl peptidase IV, also known as CD26).

In another embodiment the antibodies of the present invention specifically bind invadopodia of cells of a tissue undergoing angiogenesis. Such cells may be cancerous cells, cells of a tumor in a human being *in vivo,* or the cells may be cells may comprise a tissue or an organ undergoing an *ex-vivo* procedure.

The active antibodies and active antibody fragments of the present invention exhibit one or more of the following characteristics:

i) the antibodies specifically bind to the invadopodia of invasive cells grown in collagen or on fibronectin films,

ii) the antibodies antibody fragments fail to react with non-invasive human carcinoma cells grown in collagen or on fibronectin films.

iii) the antibodies antibody fragments bind weakly to differentiated human endothelial cells in collagen or matrix gels and more strongly to

sprouting human endothelial cells in collagen or matrix gels,

iv) the antibodies antibody fragments bind weakly with connective tissue cells and more strongly with these induced by wounding,

v) the antibodies antibody fragments block the interaction of collagen matrix with reactive human cells and inhibit the collagen degradation by such cells and

vi) the antibodies or antibody fragments react readily with the catalytic or substrate binding domains of DPPIV and of the seprase-DPPIV complex.

The antibodies of the present invention may comprise a seprase-DPPIV antagonist for use in the present methods. Such seprase-DPPIV antagonists are capable of binding to the catalytic and substrate-binding domains and competitively inhibiting the ability of seprase-DPPIV to interact with a natural ligand such as type I or IV collagen. Preferably, the antagonist exhibits specificity for seprase and DPPIV over other proteases, including urokinase and matrix metalloproteases. In a particularly preferred embodiment, a polypeptide or antibody fragment react readily with the catalytic or substrate-binding domains of the seprase-DPPIV complex and inhibits binding of collagen or E19 and E26 monoclonal antibodies to said domains. A preferred seprase-DPPIV antagonist may be a polypeptide or a monoclonal antibody, or functional fragment thereof, that is immunoreactive with either the catalytic or substrate-binding domains (or both) of the seprase-DPPIV complex. In one embodiment the antibody competitively inhibits the peptidase activity of DPPIV.

Also provided by the present invention are bispecific antibodies with binding specificity for two epitopes, one of which is an epitope of DPPIV. The bispecific antibodies of the present invention include those in which the second epitope bound is an epitope of seprase, MT1-MMP, MMP-2 or an integrin, such as $\alpha(3)\beta(1)$-integrin, $\alpha_v\beta_3$-integrin or $\beta 1$-integrin. The second epitope may be an epitope of any tumor-associated antigen.

The present invention further provides immunoconjugates comprising a monospecific antibody which specifically binds an epitope of human DPPIV and inhibits angiogenesis, joined to a therapeutic agent. These immunoconjugates include those which comprises the monoclonal antibodies E19 or E26 or fragments of such

antibodies. In a preferred embodiment the immunoconjugate is capable of killing cells involved in angiogenesis.

Alternatively the immunoconjugates of the present invention may include recombinant, chimeric, or humanized antibodies; or fragments of any of these. The immunoconjugates of the present invention may comprise a therapeutic agent such as an anti-tumor drug, a cytotoxin, a radioactive agent, a photosensitizer, a second antibody or an enzyme.

In yet another aspect the invention provides pharmaceutical compositions for inhibiting angiogenesis comprising an effective amount of a monospecific antibody which specifically binds an epitope of a human DPPIV and inhibits angiogenesis, the antibody being formulated in a pharmaceutically acceptable carrier.

The invention further provides pharmaceutical compositions for inhibiting angiogenesis comprising an effective amount of a bispecific antibody which specifically binds an epitope of a human DPPIV and inhibits angiogenesis, the antibody in a pharmaceutically acceptable carrier.

In yet another aspect the invention provides pharmaceutical compositions for inhibiting angiogenesis comprising an effective amount of an immunoconjugate of a monospecific or a bispecific antibody which specifically binds an epitope of a human DPPIV and inhibits angiogenesis, the antibody being formulated in a pharmaceutically acceptable carrier.

The present invention yet further provides a method of treating a patient suffering from a growth or proliferative disorder involving angiogenesis, comprising administering an effective amount of a monospecific antibody which specifically binds an epitope of a human DPPIV and inhibits angiogenesis.

The present invention also provides a method of treating a patient suffering from a growth or proliferative disorder involving angiogenesis, comprising administering to the patient an effective amount of a bispecific antibody which specifically binds an epitope of a human DPPIV and inhibits angiogenesis.

In yet another aspect the present invention provides a method of treating a patient suffering from a growth or proliferative disorder involving angiogenesis, comprising administering an effective amount of an immunoconjugate of a monospecific antibody which specifically binds an epitope of a human DPPIV and

inhibits angiogenesis.

The present invention further provides a method of treating a patient suffering from a growth or proliferative disorder involving angiogenesis, comprising administering an effective amount of an immunoconjugate of a bispecific antibody which specifically binds an epitope of a human DPPIV and inhibits angiogenesis.

The anti-angiogenic treatment methods of the invention described above may be applied to patients with solid tumors, preferably to inhibit angiogenesis and metastasis, and most preferably to induce tumor regression. More preferably still, the treatment methods are capable of curing the patient of the tumor such that tumor regression is substantially complete.

Also provided are continuing hybridoma cell lines, which secrete recoverable quantities of monoclonal antibodies which specifically bind an epitope of a human DPPIV and inhibit angiogenesis. In a particular embodiment the hybridoma is one that produces a monoclonal antibody of the class IgG_{2a}, designated E19. In another particular embodiment the hybridoma is one that produces a monoclonal antibody of the class IgG_{2a}, designated E26.

The invention relates to a membrane protease complex, consisting of two homodimers of seprase and dipeptidyl peptidase IV (DPPIV), initially obtained from human placental capillary endothelial membranes, monoclonal antibodies against components of the complex and a method of inhibiting capillary sprouting and angiogenesis in human cancer.

Two novel rat monoclonal antibodies of the class IgG.2a react readily with the protease complex consisting of seprase and DPPIV that resides on cell surface invadopodia at the leading edge of angiogenic endothelia, migratory fibroblasts, and invading cancer cells. These antibodies fail to react with resting cells in adjacent human tissues and blood vessels. They also block interaction of collagen matrix with the seprase-DPPIV complex in the invasive cells during angiogenesis and cancer spreading but not that with other endothelia or tumor cells not undergoing angiogenesis.

The disclosures of the present invention herein demonstrates that angiogenesis in tissues requires a membrane protease complex, consisting of two homodimers of seprase and dipeptidyl peptidase IV (DPPIV), and that inhibitors of the seprase-

DPPIV complex can inhibit angiogenesis. The disclosure also demonstrates that antagonists of two novel rat monoclonal antibodies of the class IgG.2a react readily with the catalytic and substrate-binding domains of the protease complex that resides on cell surface invadopodia at the leading edge of angiogenic endothelia, migratory fibroblasts, and invading cancer cells, while failing to react with resting cells in adjacent human tissues and blood vessels. These antibodies have the property of blocking interaction of collagen matrix with the seprase-DPPIV complex in the invasive cells during angiogenesis and cancer spreading but not that with other endothelia or tumor cells.

The invention describes methods for inhibiting angiogenesis and cancer metastasis in a mammalian tissue comprising administering to the mammal a composition comprising an effective amount of a seprase-DPPIV antagonist such as monoclonal antibody E19 or E26.

The methods of the present invention are applicable to any mammal. Mammals include, for example, laboratory animals such as rats, mice and guinea pigs; farm animals such as cows, horses, sheep and goats; pet animals such as dogs and cats; primates such as apes and monkeys; and most preferably, human patients.

The tissue to be treated can be any tissue in which inhibition of angiogenesis or cell invasion is desirable, such as diseased tissue where neo-vascularization or cancer spreading is occurring. Exemplary mammalian, particularly human tissues include various types of carcinomas, metastases, tissues undergoing restenosis, inflamed tissue, and the like.

A seprase-DPPIV antagonist for use in the present methods is capable of binding to the catalytic and substrate-binding domains and competitively inhibiting the ability of seprase-DPPIV to interact with a natural ligand such as type I or IV collagen. Preferably, the antagonist exhibits specificity for seprase and DPPIV over other proteases, including urokinase and matrix metalloproteases. In a particularly preferred embodiment, a polypeptide or antibody fragment react readily with the catalytic or substrate-binding domains of the seprase-DPPIV complex and inhibits binding of collagen or E19 and E26 monoclonal antibodies to said domains. A preferred seprase-DPPIV antagonist can be a polypeptide or a monoclonal antibody,

or functional fragment thereof, that immunoreacts with the catalytic or substrate-binding domains of the seprase-DPPIV complex.

Seprase and DPPIV are activated on specialized protrusions (invadopodia) of migratory endothelial cells. Both seprase and DPPIV are transiently expressed in endothelial cells at sites of sprouting vessels but not in differentiated vessels in human breast cancer tissue or in human angiogenesis models. In contrast, other known targets for anti-angiogenesis therapies which include β1-integrins, MT1-MMP and MMP-2 are constitutively expressed in endothelial cells. Antibodies to DPPIV block endothelial migration and sprouting but do not affect preexisting capillaries; whereas β1-integrin antibodies or MMP inhibitors strongly disturb both processes. Because seprase and DPPIV are co-expressed at very low levels in differentiated endothelium, they make attractive new therapeutic targets for cancer angiogenesis.

In another aspect, the invention provides a method of stimulating angiogenesis in a mammal suffering from disease or disorder that may be remedied by an increased blood supply. The disease or disorder may be any disease or disorder in which the blood supply in at least one tissue is reduced or restricted. There are many such diseases and disorders. For instance, one such disease is cardiovascular disease, in which the blood supply to heart muscle tissue may be reduced or severely restricted. The method provided by the present invention causes stimulation of angiogenesis in the afflicted mammal by administering an angiogenesis-stimulating amount of a DPPIV stimulator, thereby increasing the blood supply to the affected tissue.

A DPPIV stimulator, according to the present invention is any compound that enhances the activity or expression of the DPPIV peptidase. Stimulation caused by the DPPIV stimulator may be by any mechanism. This may be by stimulation of the activity of DPPIV by the DPPIV stimulator acting as an agonist. Alternatively, the stimulation may be achieved by increased expression of the DPPIV peptidase.

EXAMPLES

Methods: Following methods were carried out as described: immunohistological staining of tissue sections (Kelly et al., 1998); seprase/DPPIV protein and proteolytic

activity (Pineiro-Sanchez et al., 1997); RT-PCR (Goldstein et al., 1997); double-labeled immunofluorescence of cultured cells and β1-integrin blotting (Mueller et al., 1999), MMP-2 activity (Nakahara et al., 1997); endothelial migration and monolayer wound assays (Pepper et al., 1996); HUVEC (Human umbilical vein endothelial cell) culture and Matrigel tube assay (Grant et al., 1992).

Example 1: Cytoimmunohistochemical staining for seprase and DPPIV in endothelial cells sprouting vessels and in normal tissue. To investigate the expression of seprase and DPPIV during angiogenesis, human malignant breast carcinoma tissue or adjacent normal skin were stained with antibodies specific for either seprase or DPPIV. Both seprase and DPPIV were abundantly expressed on the endothelial cells of sprouting vessels (Fig. 1, solid arrows) but were not detectable in other tumor vessels (Fig. 1, open arrows) or in adjacent normal skin from the same donor. These findings indicate that only sprouting sites of blood vessels involved in tumor angiogenesis have enhanced expression of seprase and DPPIV. Consistent with this result, expression of seprase and DPPIV on cultured endothelial cells can be induced by means or factors that enhance cell migration and vessel sprouting (see below).

Example 2: Cytoimmunohistochemical staining for seprase and DPPIV in human primary cell culture monolayers of different cell densities. Endothelial cells of sprouting vessels are migratory and exhibit a lack contact inhibition (Pepper et al., 1993). Monolayer cultures of human umbilical vein endothelial cells (HUVEC) can be induced to migrate by wounding or passage to low cell density (Pepper et al., 1996). This assay was used to examine the expression of seprase and DPPIV in migratory endothelial cells. The confluent HUVEC monolayers were found to contain low levels of seprase and DPPIV and their proteolytic activities were also low (Fig. 2a-c). Passage of monolayers into a sparse culture within 24 hours induced the expression of functional seprase protein; it also caused an increase in DPPIV protein and their proteolytic activities (Fig. 2a-c).

Both HUVEC cultures had detectable seprase and DPPIV mRNA (Fig. 2d-e). As β1-integrins (Bloch et al., 1997) and membrane-bound MMP-2 (Hiraoka et al., 1998) have been shown to be essential for angiogenesis, we examined their presence in this assay. β1-integrin and MMP-2 gelatinase activity were readily detectable in both confluent and sparse endothelial cultures, while seprase and DPPIV (protein/activity) were increased in the sparse culture (Fig. 2a-b). The result strongly suggests the association of seprase and DPPIV expression with migratory activity of endothelial cells.

Example 3: Expression of seprase/DPPIV and β1-integrins during wound-induced endothelial migration. The relative expression of seprase/DPPIV and β1-integrins during wound-induced endothelial migration was examined by double-labeled immunofluorescence. The monolayer wound model consists of a 300 μm width wound on the HUVEC monolayer; migratory activity of HUVEC was visible one hour after wounding (Fig. 2f-g). Expression of β1-integrins was high in endothelial cells at both the wound edge (wounded) and in the monolayer (stationary); in contrast, seprase and DPPIV expression was restricted to migratory cells at the wound edge (Fig. 2f-g, arrows).

In addition, seprase and DPPIV expression was found on invadopodia (Fig. 2f-g, arrows) and on the perinuclear region (Golgi apparatus) of migratory cells but not in confluent cells. In phase contrast images (Fig. 2f-g), β1-integrins were distributed widely on the surface of migratory cells (Fig. 2f-g, solid arrows) and particularly concentrated at sites of contact between confluent cells, suggesting the role of integrins in both cell migration and adhesion. Similar to β1, membrane type-1 MMP was found distributed evenly on the cell surface of HUVEC at the wound edge and in stationary monolayers.

Example 4: Inhibitory effects of anti-DPPIV mAbs on cell migration of fibroblasts and of wounded cells from a monolayer. The effect of various mAbs against DPPIV (E19 and E26 are inhibitory; E3 is not), β1-integrins (C27 and 13 are

inhibitory), and a cell surface glycoprotein gp90 (C37 is not inhibitory) (Mueller et al., 1999) were examined to determine whether DPPIV (and possibly seprase) plays an active role in endothelial migration. Both anti-DPPIV and anti-β1 mAbs blocked endothelial cell migration, whereas the mAb against cell surface proteins gp90 had no effect (Fig. 2h-j). Identical results were obtained when fibroblast migration and cell surface collagen degradation were induced by monolayer wounding.

Example 5: Inhibitory effects of anti-DPPIV mAbs on blood vessel tube formation. To examine the effects of these same antibodies on endothelial tube formation by Matrigel (Grant et al., 1992), mAbs or the matrix metalloprotease inhibitor CT1847 (Zucker et al., 1998) were added prior to or after tube formation (Fig. 3a). The inhibitory anti-DPPIV and β1 mAbs and CT1847 blocked tube formation in Matrigel (Fig. 3b); however, only anti-β1 mAb and CT1847 but not the anti-DPPIV mAb perturbed preexisting tubes (Fig. 3c). None of the other mAbs to DPPIV and seprase affected preexisting endothelial tubes. Specific expression of seprase and DPPIV in the endothelial cell migrated from a forming tube (Fig. 3d) also supports the observation that the anti-DPPIV mAb appears to act selectively on new tube formation.

Example 6: Effects of anti-DPPIV mAbs on invasion and capillary sprout formation of human dermal microvascular endothelial cells (HDMEC). In a recently developed *in vitro* human angiogenesis model, invasion and capillary sprout formation of HDMEC can be induced in fibrin gels in response to vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). This assay was used to determine whether DPPIV plays an active role in human angiogenesis. HDMEC were cultured on microcarrier beads and embedded in a three dimensional human fibrin gel that contained VEGF and bFGF. In this model, HDMEC formed capillary sprouts [within 24 hours] (Fig. 4a, upper pictures labeled with VEGF/bFGF+ or -). The presence of a capillary lumen was confirmed by confocal microscopy.

When the fibrin gel was impregnated with the inhibitory anti-DPPIV or β1 antibodies or CT1847, capillary sprouting was blocked (Fig. 4a-b).

Example 7: The active domain of the Seprase-DPPIV complex. Recent cloning studies (Goldstein et al., 1997) show that seprase carboxyl terminus contains a putative catalytic region (~200 amino acids), which is homologous (68% identity) to that of the non-classical serine peptidase DPPIV. The conserved serine protease motif G-X-S-X-G is present as G-W-S-Y-G. Like DPPIV, seprase have 12 Cys with 6 residues being conserved in the Cys rich region and 3 in the catalytic region. Seprase has a peculiar protease inhibitor profile: it is inhibited by the protease inhibitors, including PMSF and NEM (Aoyama and Chen, 1990). Its gelatinase activity was completely blocked by the serine-protease inhibitors, DFP, PMSF, AEBSF, and APSF. Dimeric seprase could be affinity-labeled by [^{3}H]-DFP but the proteolytically inactive 97-kDa subunit could not (Pineiro-Sanchez et al., 1997). The inhibitor and substrate specificity of the seprase-DPPIV complex isolated from human breast carcinoma cells was analyzed by [^{3}H]-DFP labeling. The method is extremely sensitive in detecting serine proteases and esterases (10^{-13} M) and is based upon the stoichiometrical, covalent binding of [^{3}H]-DFP into the proteases that are reduced in the presence of their substrates and inhibitors. Both dimeric seprase and DPPIV may be labeled with [^{3}H]-DFP and their molecular identity may be visualized on SDS gels (Pineiro-Sanchez et al., 1997). By incubating the seprase-DPPIV complex with [^{3}H]-DFP in the presence of their peptide- substrates or inhibitors, the protease inhibition is quantified.

Seprase and the seprase-DPPIV complex were purified from 10^{11} LOX human malignant melanoma cells or MDA-MB-436 human breast carcinoma cells that express seprase and the seprase-DPPIV complex, respectively. Cell lysates are subjected to two steps of enrichment (Triton X-114 detergent phase partitioning and WGA chromatography) and they are stored at –80°C. Purified seprase is prepared immediately prior to experimentation by immunoprecipitation of LOX WGA-binding proteins with micro-magnetic beads (about 50 nm, Miltenyi Biotec) using mAb D28 or D8. The seprase-DPPIV complex is purified from MDA-MB-436 WGA proteins

with either anti-seprase- or anti-DPPIV-monoclonal antibodies. Purified seprase and the seprase-DPPIV complexes are used to define inhibitor-specificity and substrate-specificity of the enzymes.

Example 8: Substrate specificity of the seprase-DPPIV complex that was purified by monoclonal antibodies. Collagen-substrate specificity of the seprase-DPPIV complex was determined by incubating fluorescently labeled type I collagen with isolated seprase, DPPIV or seprase-DPPIV complex in the presence of SIMP inhibitors (PMSF inhibits seprase activity and it can be used as control). Briefly, fluorescently labeled collagen was incubated with seprase, DPPIV or seprase-DPPIV complex immobilized on mAb-beads at 37°C, in the presence or absence of enzyme inhibitors. The cleavage site of type I collagen by isolated seprase, DPPIV or seprase-DPPIV was examined. Rates of cleavage and fragment sizes was analyzed by SDS-PAGE as shown in a previous paper (Pineiro-Sanchez et al., 1997). Cleavage products, transferred to an Immobilon-P membrane was subjected to limited sequence analysis to determine the primary cleavage site(s) (Pineiro-Sanchez et al., 1997). The seprase-cleavage peptides include proline and hydroxyproline.

Example 9: X-Proline dipeptide bonds as cleavage sites of seprase and seprase-DPPIV complex. Amino acid cleavage site of individual seprase or the seprase-DPPIV complex were identified to be at X-proline dipeptide bond using method described in a previous paper (Pineiro-Sanchez et al., 1997). Classical DPPIV activity using the fluorescent Ala-Pro-AFC substrate overlay assay showed that SDS-denatured seprase or the protease complex exhibited little activity. However, native seprase and the protease complex purified by monoclonal antibodies from LOX or MDA-MB-436 or Hs578T tumor cells show strong activity toward glycine-proline or alanine-proline etc (X-proline) dipeptide bonds. See figures 5 and 6 and the descriptions of the figures for details. In addition, the figures show that mAbs E19 and E26 interfered with the glycine-proline cleavage by LOX- or Hs578T-seprase complex purified by monoclonal antibodies.

Seprase degrades denatured collagens that contain high levels of proline peptides. Its catalytic domain sequence is highly homologous to that of the proline-specific exo-peptidase DPPIV. Seprase and the seprase-DPPIV complex cleaves proline-peptide bonds including for example the following:

(omega-*N*-(*O*-acyl)hydroxy amid) aminodicarboxylic acid pyrrolidides (Demuth et al., 1993) and substrates containing phosphorylated residues adjacent to proline (Kaspari et al., 1996), which are potent inhibitors of proline-specific peptidases.

H-Ile-Pro-NHO-pNB, irreversible suicide DPPIV inhibitor
H-Ile-Thia, reversible DPPIV inhibitor.(Ki = $8x10^{-8}$)
H-Glu(NHO-Bz)-Pyrr, reversible DPPIV inhibitor (Ki = $5x10^{-7}$)
H-Glu(Gly5)-Thia, reversible DPPIV inhibitor (Ki = $8x10^{-8}$)
H-Pro-Ile-Thia, reversible PEP inhibitor
pGlu-Ile-Thia, reversible PEP inhibitor
Boc-Ile-Pyrr, reversible PEP inhibitor
Boc-Glu(NHO-Bz)-Pyrr, reversible, slowly acylating PEP inhibitor
Z-Phe-Ala-CMK, irreversible PEP inhibitor
Z-Gly-Pro-AMC, fluorgenic PEP substrate
H-Gly-Pro-AMC, fluorogenic DPPIV substrate.

Thus, small or large molecules interfering the interaction of seprase or the protease complex with X-proline in collagen have inhibitory activity for the seprase-DPPIV complex.

Example 10: Phage displayed peptides that recognize specific sites of the seprase-DPPIV complex that was purified by monoclonal antibodies. Small peptides that specifically recognize the active sites of the seprase-DPPIV complex are identified using libraries of phage display peptides originally designed by George Smith (Scott and Smith, 1990). Using seprase or the seprase-DPPIV complex purified by mAbs E19 and D8, tens of billions of short peptides are screened and selected for tight binding to specific proteases. The library is a mixture of a vast number of filamentous phage clones, each displaying one peptide sequence on the virion surface.

The selection is accomplished by using the above protease preparations to affinity-purify phages that display tight-binding peptides and propagating the purified phage in *E. coli*. The amino acid sequences of the peptides displayed on the phage are then determined by sequencing the corresponding coding region in the viral DNAs. Specific peptides, displayed on filamentous phages that (i) bind to isolated enzyme or proteins, (ii) block enzymatic activity, and (iii) bind to inhibitory antibodies E19 and E26 against DPPIV are selected.

Such peptides that are recognized by monoclonal antibodies E19 or E26 may lead to the identification of a novel epitope involved in activity of the protease complex. For panning on monoclonal antibodies in (iii) above, inhibitory protease complex mAbs E19 and E26 are used to screen peptides from a random peptide library of 15 amino-acid residues as the approach previously used in the identification of human hepatitis B virus surface epitopes (Motti et al., 1994). The peptides recognized by the mAbs are analyzed for their amino acid similarity to the natural protease antigens, and the selected phage-displayed epitopes behave as antigenic mimics.

Example 11: Identification of substrates for the seprase-DPPIV complex using bacteriophage peptide display libraries and monoclonal antibody purification of the protease complex. Potential peptide substrates for seprase or the seprase-DPPIV complex are also identified using bacteriophage peptide display libraries that have been used by Navre's group to identify peptide substrates for stromelysin and matrilysin (Smith et al., 1995). The random hexamer library in the fd-derived vector fAFF-1 included a "tether" sequence that is recognized by monoclonal antibodies. The phage library is treated in solution with seprase or the seprase-DPPIV complex. Cleaved phage is separated from uncleaved phage using a mixture of tether-binding monoclonal antibodies and Protein A-bearing cells followed by precipitation. Clones are screened by the use of a rapid "dot-blot-proteolysis" assay as described in the above reference that identifies phage encoding peptide sequences susceptible to cleavage by the enzyme. The nucleotide sequence of the random hexamer region of isolated clones are determined. Synthetic peptides th n are prepared whose sequences

are based on some of the positive clones, as well as consensus sequences built from the positive clones. Seprase or specific seprase-DPPIV substrates that are both the most active and smallest are selected. The peptide substrates are used to conjugate with fluorescent AMC, which in turn will be used in search for potential inhibitors using other phage peptide display libraries.

Example 12: General Procedure for Conjugating Small Molecular Drugs to an Antibody. Antibody-small molecule conjugates are prepared by linking the DOX derivative maleimidocaproyl doxorubicin hydrazone or the maleimidocaproylhydrazone of Adriamycin to E19, E26 or control immunoglobulin following the procedure of Hellstrom, US Patent No., 5,980,896. Antibody is diluted with 0.0095 M PBS to a protein concentration of 10.49 mg/mL. This solution (500 mL) is heated to 37°C., under a nitrogen atmosphere, in a water bath. Dithiothreitol (26.2 mL, 10 mM) in PBS is added and the solution is stirred for 3 hrs at 37°C. The solution is divided equally between two Amicon Model 8400 stirred ultrafiltration cells each fitted with a YM 30 ultrafilter (MW cutoff 30,000, 76 mm diam.) and connected via a Model CDS10 concentration/dialysis selector to a Model RC800 mini-reservoir (Amicon, Division of W. R. Grace and Co., Beverly, MA). Each reservoir contains 800 mL of 0.0095 M PBS-0.1 M L-histidine. The protein solutions are dialyzed until the concentration of free thiol in the filtrate was 63 .mu.M. The molar ratio of --SH/protein in the retentate is determined to be 8.16. The retentate is transferred from the cells to a sterile container under nitrogen and a solution of maleimidocaproyl hydrazone of adriamycin (42.6 mL, 5 mg/mL in water) is added with stirring. The conjugate is incubated at 4°C. for 48 hrs after which it is filtered through a 0.22.mu. cellulose acetate membrane. A 2.5 cm.x.50 cm Bio-Rad Econocolumn is packed with a slurry of 100 g of BioBeads.TM.SM-2 (Bio-Rad Laboratories, Richmond Calif. 94804) in 0.0095 M-0.1 M L-histidine buffer. The beads are prepared by washing in methanol, followed by water then several volumes of buffer. The filtered conjugate is percolated through this column at 2 mL/min. After chromatography the conjugate is filtered through a 0.22.mu. cellulose acetate membrane, frozen in liquid nitrogen and stored at -80°C. The conjugate obtained has a molar ratio of 6.77 Adriamycin to protein and is obtained in 80-95% yield.

Example 13: Biological Activity of conjugates. Representative conjugates of the present invention are tested in both in vitro and in vivo systems to determine biological activity. The potency of conjugates of cytotoxic drugs is determined by measuring the cytotoxicity of the conjugates against cells of human cancer origin. The following describe representative tests and results. The conjugates are referred to using the form ligand-drug-molar ratio of ligand to drug.

Experimental Human Angiogenesis Assay.

The assay system measures human angiogenesis, invasion and metastasis in the chimeric mouse:human model and is referred to as the experimental human angiogenesis assay. The assay has been described in detail by others, and further has been described herein to measure human angiogenesis, invasion and metastasis. See (Yan et al., 1993). Yan, et al., J. Clin. Invest., 91:986-996 (1993).

The experimental human angiogenesis assay is a useful assay model for in vivo angiogenesis because the transplanted skin grafts closely resemble normal human skin histologically. In this model, human cancer cell invasion and neovascularization are occurring wherein actual human blood vessels and tissue are growing from the grafted human skin into the human tumor tissue on the surface of the grafted human skin. The origin of the neovascularization into the human graft can be demonstrated by immunohistochemical staining of the neovasculature with human-specific endothelial cell markers. The invasion and metastasis of human cancer cells may be monitored also.

As demonstrated herein, the experimental human angiogenesis assay demonstrates regression of neovascularization based on both the amount and extent of regression of new vessel growth. Furthermore, effects on the invasion and metastasis of any cancer tissue transplanted upon the grafted skin are easily monitored. Finally, the assay is useful because there is an internal control for toxicity in the assay system. The SCID mouse is exposed to any test reagent, and therefore the health of the mouse is an indication of toxicity.

The experimental human angiogenesis model is prepared essentially as described in Yan, et al., J. Clin. Invest., 91:986-996 (1993). Briefly, a 2 cm^2 square area of skin is surgically removed from a SCID mouse (6-8 weeks of age) and replaced with a human foreskin. The mouse is anesthetized and the hair removed from a 5 cm^2 area on each side of the lateral abdominal region by shaving. Two circular graft beds of 2 cm^2 are prepared by removing the full thickness of skin down to the fascia. Full thickness human skin grafts of the same size derived from human neonatal foreskin are placed onto the wound beds and held in place with 5-0 monofilament suture (Dermalon, Davis and Geck Inc., Danbury, CT). The graft is covered with a Band-Aid, which is sutured to the skin. Micropore surgical tape (3M, St. Paul, MN) is also applied to cover the wound. Mice are housed in individual cages.

The LOX human melanoma cell line that expresses only seprase (Fodstad et al., 1988) or MDA-MB-436 breast carcinoma cell line that expresses the seprase-DPPIV complex (ATCC HTB 130), as determined by immunoreactivity of the cells with mAb D28 (anti-seprase) and E3 (anti-DPPIV), are used to form the solid human tumors on the human skin grafts on the SCID mice. A single cell suspension of 5 x 10^5 LOX or MDA-MB-436 cells is injected intradermally into the human skin graft. The mice are then observed for 2 to 4 weeks to allow growth of measurable human tumors.

Following the growth of measurable tumors, SCID mice, which had been injected with LOX or MDA-MB-436 human tumor cells, are injected intravenously into the tail vein with 250 .u.g of either the mAb E19/26 (anti-complex, inhibitory) or E3 (anti-DPPIV, non-inhibitory) twice a week for 2 to 3 weeks. After this time, the tumors are resected from the skin and trimmed free of surrounding tissue. Several mice are evaluated for each treatment with the average tumor weight from each treatment being calculated.

Exposure of the LOX- or MDA-MB-436-seprase complex positive human carcinoma tumor mass in the experimental human angiogenesis model to E19 or E26 (against the active site) causes the decrease from the non-inhibitory mAb E3 treated average tumor weight reduction of 100 mg. Representative examples of LOX tumors

treated with the mAb E19 and E3 are examined morphologically. The E3-treated tumors remain large (8 to 10 mm in diameter) and well vascularized whereas those treated with mAb E19 (against the active site) are much smaller (3 to 4 mm in diameter) and lack detectable blood vessels. Thus, the blocking of the seprase-DPPIV complex by the intravenous application of active site-specific E19 or E26 antibodies results in a regression of a human melanoma or carcinoma in this model system.

The foregoing results demonstrate that the membrane-bound serine protease DPPIV and possibly also seprase, play a key role in endothelial migration and sprouting but not capillary stability. In addition, the findings that seprase/DPPIV is expressed specifically at sites of capillary sprouts, and that antibodies to DPPIV inhibited endothelial cell migration and capillary sprouting in various extracellular matrices, without perturbing preexisting capillaries, are consistent with the functioning of these proteases in angiogenesis.

Furthermore, DPPIV has been shown to be a gelatinase (Bermpohl et al., 1998) as well as an adhesion receptor for collagen (Bauvois, 1988; Hanski et al., 1988; Loster et al., 1995) or fibronectin (Cheng et al., 1998; Piazza et al., 1989; Johnson et al., 1993); whereas seprase, originally identified as a 170 kDa membrane-bound gelatinase, also associates with the adhesion receptor α3β1-integrin (Aoyama and Chen, 1990; Mueller et al., 1999). Thus, seprase and DPPIV are specific targets for capillary sprouting.

Example 14: Monoclonal Antibodies: The rat monoclonal antibodies (mAbs) E26, E19, E3 and F4 are directed against human placental DPPIV according to the method of production of rat mAbs D8 and D28 against human placental seprase as described previously (Pineiro-Sanchez et al., 1997; Goldstein et al., 1997). The seprase-DPPIV complex had been isolated from human placenta, and antibodies were produced as described (Pineiro-Sanchez et al., 1997). Monoclonal antibodies E26, E19, E3 and F4 react with DPPIV of the seprase-DPPIV complex, and are not immunoreactive with the seprase subunit or with serine integral membrane proteinases (SIMPs).

Example 15: Cancer cell lines with altered levels of DPPIV or seprase. The LOX human melanoma cell line (Fodstad et al., 1988) or MDA-MB-436 breast carcinoma cell line (ATCC HTB 130) that express seprase and the seprase-DPPIV complex, respectively, as determined by immunoreactivity of the cells with mAb D28 (anti-seprase) and E3 (anti-DPPIV), were used to study the role of DPPIV and seprase. For this project, the specific importance of DPPIV and the DPPIV-seprase complex against the MT1-MMP background in cancer invasion and angiogenesis were defined using over-expression of DPPIV in low expresser MDA-MB-231 cells (seprase-/DPPIV-/MT1-MMP+) or LOX malignant melanoma cells (seprase+/DPPIV-/MT1-MMP+), alternatively, anti-sense and ribozyme knockout of mRNA in high expresser MDA-MB-436 (seprase+/DPPIV+/MT1-MMP+) as shown in Fig. 7. Inhibition of protease activity by monoclonal antibodies was related to these cell transfection results.

The characterization of protease expression has been completed in selected human breast carcinoma and melanoma cell lines as well as in a pilot study using 70 cases of invasive ductal carcinoma and their respective, adjacent "normal" tissues. RT-PCR was used to investigate the presence of mRNA, Western blotting for relative protein expression levels, gelatin zymography and Ala-Pro-AMC substrate overlay assay for seprase and DPPIV enzymatic activities, respectively, immunofluorescence for surface localization at invadopodia, and immunohistochemistry (IHC) for cell type-distribution of seprase and DPPIV, as well as endothelial and epithelial marker stainings. In summary, the involvement of seprase, DPPIV and MT1-MMP was found at invadopodia. However, two moderately invasive human breast carcinoma cell lines were identified: a seprase and DPPIV low expresser MDA-MB-231 (seprase-/DPPIV-/MT1-MMP+) and a seprase and DPPIV high expresser MDA-MB-436 (seprase+/DPPIV+/MT1-MMP+) as shown in Fig. 7. These two cell lines offer an opportunity to examine relative contribution of seprase and DPPIV, against the background of MT1-MMP in cell invasiveness.

Studies abrogating seprase or DPPIV expression in invasive cells that expressed these proteases constitutively by antisense and ribozyme constructs have been performed. Initially, anti-sense and ribozyme methods were applied to the

moderately invasive breast carcinoma cell line, MDA-MB-436 (seprase+/DPPIV+/MT1-MMP+). A stable transfectant of the MDA-MB-436 parent line has been isolated, which has integrated a recombinant expression vector (pCR3.1) containing seprase cDNA in the antisense orientation into its genome (pA26). The cells suppress the seprase expression. The ability of the cells to degrade collagen fiber is partially reduced (Fig. 8). A ribozyme construct (rbz8C#1) according to the method of ribozyme knockout of mRNA (Haseloff and Gerlach, 1988) has been assembled. The ribozyme exhibits *in vitro* cleavage activity against a DPPIV transcript at the selected site. Fig. 8 shows that the ribozyme blocks not only DPPIV but also seprase protein expression in MDA-MB-436 cells as demonstrated by Western blotting. The stable MDA-MB-436 cell line that had DPPIV RNA-ribozyme knockout partially blocked the degradation of collagen matrix by the cells. It was surprising that the DPPIV-ribozyme cells also suppressed seprase expression (Fig. 8). Furthermore, the degradation of collagen matrix by this cell line was only observed being moderate (Fig. 8), suggesting that a complicated process of regulation may occur in this protease expression system.

Using the ribozyme approach, DPPIV expression was abrogated in invasive cells to examine the influence of individual membrane proteases on tumor cell proliferation and invasiveness. A hammerhead ribozyme usually consists of a catalytic center made up of a highly conserved sequence of 22 nucleotides and two flanking regions that base-pair the ribozyme with targeted RNA sequences flanking the trinucleotide GUX, where X can be A, C or U. A ribozyme normally cleaves on the 3' side of the trinucleotide in the targeted RNA (Haseloff and Gerlach, 1988). To generate ribozyme constructs targeted at DPPIV, the cDNA sequence was scanned and potential cleavage sites identified. A pair of sense and anti-sense oligonucleotides with complimentary 3' ends, covering the sequences of the catalytic center and flanking regions of the cleavage site have been synthesized. As an example, the nucleotide sequences of sense and antisense oligonucleotides used to construct the ribozyme #8 (rbz#8) targeting a site in the seprase sequence are shown as follows:

5'-AGGCACTGAACTGATGAGTCCGTGAGG-3' Sense oligonucleotide

5'-TGAAGAGGAAGTTTCGTCCTCACGG-3' Antisense oligonucleotide

The above sequences of sense and antisense oligonucleotides were used to construct a ribozyme targeting a sequence in DPPIV. The ribozyme oligonucleotides were annealed, amplified by PCR, then cloned into pCR3.1 vector using the TA-cloning method (Invitrogen). Ribozyme constructs were screened by restriction enzyme analysis and verified by sequencing. Ribozyme rbz#8 cleaved DPPIV nucleotides at expected site and generated expected cleavage products. The rbz#8 construct demonstrating the most effective in vitro cleavage activity was selected for transfection of carcinoma cells to generate stable transfected clones. These cells were assayed as described above.

Example 16: Characterization of the DPPIV catalytic site: (a) Enzymatic assays for the DPPIV catalytic site. A commercial chromogen, Gly-Pro-p-nitroanilide (pNA), is a substrate for the catalytic domains of both DPPIV and the seprase-DPPIV complex. Inhibitors of DPPIV include compounds containing phosphorylated residues adjacent to proline, e.g., H-Ile-Pro-NHO-pNB, an irreversible suicide DPPIV inhibitor; H-Ile-Thia, a reversible DPPIV inhibitor (Ki= 8x10-8); and H-Glu(Gly5)-Thia, a reversible DPPIV inhibitor (Ki= 8x10-8) (Kaspari et al., 1996), which blocks DPPIV catalytic activity against Gly-Pro-pNA. The effect of various mAbs against DPPIV (E3, F4, E19 and E26) on the catalytic activity was examined. However, none of these antibodies block DPPIV dipeptidase activity against Gly-Pro-pNA. Interestingly, the mAbs E19 and E26 enhance DPPIV dipeptidase activity against Gly-Pro-pNA 3-5 folds but mAbs E3 and F4 do not. Thus, E19 and E26 antibodies do not have inhibitory activity against the catalytic sites as assayed by these small peptide substrates. However, binding of mAbs E19 and E26 to DPPIV may modify the proteolytic activity of the enzyme.

(b) Antibody inhibition of DPPIV substrate sites that bind to type I collagen. Collagen-degrading activity of isolated DPPIV or seprase-DPPIV complex was

determined by the release of peptide fragments from biotinylated type I collagen gel by immuno-isolated proteases or the protease complex. Fig. 9 shows the peptides released from the collagen gel in the presence of DPPIV or protease complex and potential inhibitors. The reaction was performed at 37°C for 8 h, during which time collagen gels in protein controls (Fig. 9, lanes marked Control and Ip β3 integrin) remained in gel form and relatively intact. However, when the reaction was performed at 37°C for 8-24 h, properties of denatured collagen were seen, i.e., auto-released collagenous peptides with more accessibility to MMP-2 digestion (Fig. 9). This confirms that the degradation of collagen by the seprase-DPPIV complex is due to gelatinolytic activity of the enzymes. The seprase-DPPIV complex, immuno-isolated by anti-seprase or DPPIV antibodies, exhibited a gelatinolytic activity that released multiple peptide fragments from the collagen gel, which differed from that by MMP-2 (Fig. 9). The collagen peptide pattern generated by the seprase-DPPIV complex appears to be due to a close cooperation between seprase and DPPIV in the protease complex, and the degradation is blocked by inhibitory mAbs E19 and E26 and the serine-proteinase inhibitor AEBSF, but not by the metallo-proteinase inhibitor CG1847 (Fig. 8). In addition, uncomplexed seprase derived from LOX melanoma cells and uncomplexed DPPIV isolated from bovine kidney brush border membranes did not show such collagen degrading activity. Thus, the DPPIV-seprase complex exhibits an extensive gelatin-degrading activity, and E19 and E26 inhibition of the degradation may result from antibody effects on binding of collagen substrates to the enzymes.

To map epitopes recognized by mAbs E19 and E26 at substrate binding sites for type I collagen, a competition assay was performed. A 96-well microtiter plate was coated with rat anti-DPPIV mAbs E3 or F4 and incubated at 37oC for 2 hours. The cell lysates containing the seprase-DPPIV complex, recombinant seprase or DPPIV were added and incubated at 37 oC for 2 hours. Biotinylated type I collagen, 5 μg/ml, was added in the presence of various concentrations, from 0.1 to 50 μg/ml, of mAbs E19, E26, E3 or F4. Following incubation and washing, Alkaline Phosphatase (AP) conjugated streptavidin was added to wells, and the reaction was detected by adding AP substrate (p-nitrophenylphosphate) and reading the absorbance at 405nm. E19

and E26 antibodies but not E3 and F4 antibodies recognize specific sites on DPPIV and the DPPIV-seprase complex that bind to type I collagen.

The possibility that DPPIV functions as an adhesin receptor was examined by investigating the inhibitory effect of mAbs E19 or E26 (against DPPIV) as compared to that of mAb C27 (against β1 integrins) on cellular attachment and spreading on a collagen substratum. In a parallel comparison to integrin adhesion to collagen fibers, while mAbs E19 or E26 (against DPPIV) inhibit cellular migration in collagen gels and collagen degradation by migratory cells (see below) these mAbs do not affect cellular spreading and attachment on a collagen substratum. However, mAb C27 inhibits cellular spreading on and adhesion to collagen substratum but mAbs E19 or E26 do not. These data demonstrate that whereas β1 integrins may be the primary collagen receptors on the cells responsible for cellular adhesion and spreading, DPPIV binding to collagen substrate is involved in the modification of collagenous matrices and subsequent cellular invasion.

Example 17: Characterization of tissue distribution of seprase-DPPIV complex expression. Based on immunohistochemistry (IHC) and seprase-specific gelatin zymography, seprase/FAPα expression was found to be high in mesenchymal and endothelial cells of placental tissues (Pineiro-Sanchez et al., 1997) but undetectable in human adult skin, mammary gland, gingival mucosa, and other connective tissues. Based upon mAb F19 reactivity, FAPα was originally proposed as a specific marker for "activated" fibroblasts as it was found to be expressed on reactive stromal fibroblasts of epithelial cancers and healing wounds but not in all adult tissue cells, particularly epithelial and endothelial cells (Garin-Chesa et al., 1990). Consistent with putative FAPα function in wound repair, seprase was found to be expressed transiently in mucosa cells within 1-3 days after gingival surgery. However, IHC studies for seprase expression and biochemical analysis for its function have not been done in other normal tissues and blood-borne cells, the expression and function of seprase/FAPα in normal tissue remains to be determined.

DPPIV expressions are also high in embryonic tissues and some adult tissues, including small intestine, lung and kidney. DPPIV, in specialized epithelial cells, is expressed constitutively on brush border membranes of epithelial cells of intestine and kidney, as well as the apical surface of some specialized epithelial cells, i.e., bile canaliculi of hepatocytes (Yaron and Naider, 1993; Morimoto and Schlossman, 1994). However, its transient expression on T-cells has been implicated as a marker for T-cell activation (Morimoto and Schlossman, 1994; Vivier et al., 1991). Similar to seprase, IHC studies and biochemical analysis for DPPIV function have not been done in other normal tissues and blood-borne cells, its expression and function in normal tissue remains to be determined.

To investigate the expression of the seprase-DPPIV complex during cancer invasion, angiogenesis and tissue repair, human wound granulation tissue or adjacent normal skin and infiltrating ductal carcinomas or their corresponding adjacent normal tissues were examined. Human gingival biopsies were derived from the University of Turku, Finland. Full thickness wounds of oral mucosa were made from two healthy volunteers and biopsies were collected after 3, 7, 14 and 28 days of wounding. Immediately after biopsy, fresh tissue blocks were mounted in Histoprep® (Fisher Scientific, New Jersey) and snap frozen in liquid nitrogen. Frozen sections (6 μm) were cut, fixed with acetone at -20°C for 5 min, and stored at -70°C. For routine histology, the sections were stained with hematoxylin and eosin. For immunohistochemical staining, sections were washed with PBS containing 0.1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) and incubated with rhodamine-conjugated mAb D28 against seprase or mAb E19 against DPPIV in PBS/BSA in humid chamber at 4°C for 16 h. The sections were then washed with PBS/BSA and water, briefly air-dried, and mounted using cyanoacrylate glue (Krazy Glue, Borden Company LTD). The staining was examined using a Zeiss Axioskop 20 light, fluorescence and confocal microscopy, and photographed using MC 80 Zeiss microscope camera. Control staining was performed with rhodamine-conjugated secondary antibody and showed no specific stain. Immunohistochemical staining of invasive human breast carcinoma was performed as described (Kelly et al., 1998).

Unlike human umbilical cord smooth muscle cells in culture (Goldstein et al., 1997), both seprase and DPPIV preferentially distribute among mesenchymal cells but not differentiated muscle and endothelial cells of large vessels in human embryonic tissues, including placenta and umbilical cord. To determine if seprase and DPPIV expression in stromal fibroblasts is induced during wound closure in vivo, the immunohistochemistry of human gingival mucosa-wound closure was investigated. A strong expression of seprase and DPPIV was seen in connective tissue cells at day 3 after wounding. Later, at day 7 after wounding, only a few cells in the middle of granulation tissue were reactive with the anti-seprase antibody but not the anti-DPPIV antibody. Seprase and DPPIV staining disappeared from connective tissue cells after one week and cells of normal mucosa adjacent to wounds also did not react with the antibodies against seprase and DPPIV. Furthermore, no specific reaction was seen in the fibrin clot area and epithelium. Seprase and DPPIV appear to be activation enzymes on fibroblastic cells that participate in the local degradation of connective tissue necessary for cellular migration.

Immunohistochemistry of human breast invasive carcinoma and adjacent normal tissue: The immunohistochemistry procedure involves fixation of invasive breast cancer and adjacent tissues with 4% paraformaldehyde in PBS for 2-4 h at 4°C, followed by paraffin embedding. Paraffin-embedded tissue blocks were sectioned in 4-μm-thick pieces using a microtome. The samples were adhered to glass slides (Matsunami, Tokyo, Japan) and dried at 42°C overnight. The slides were cooled and de-paraffinized through three changes of Americlear (Baxter, Deerfield, IL). Then the slides were rinsed in 100% ethanol twice and 95% ethanol twice and re-hydrated with distilled water. Antigens were retrieved by treating slides in a container covered with 10mM sodium citrate buffer, pH6.0, and heated at 120°C for 5 min by autoclave. After heat treatment, the buffer was allowed to cool down to normal temperature, and replaced with PBS. The slides were then treated with 10% normal blocking serum in PBS for 15-30 min. Blocking serum should be derived from the species in which the secondary antibody was made: normal horse serum was used for primary mouse monoclonals, and normal rabbit serum for primary rat monoclonals. Anti-seprase mAbs D8 or D28 or anti-DPPIV mAbs E19 or E26 in serum-free supernatant form

were added at a dilution of 1:10 to 1:25 to each tissue section and incubated at 4°C overnight in a humidity chamber. Mouse mAb against human endothelial cells (CD34, Cosmo Bio Co. Ltd., Tokyo, Japan) were added at a dilution of 1:50; and a mixture of three mouse mAbs, including C11 (1:400), ESA (1:50) and EMA (1:60) were used for marking epithelial cells. Bound primary antibody was then detected by streptavidin-biotin-peroxidase technique (DAKO) according to the manufacturer's instructions using diaminobenzidine (3,3'-diaminobenzidine tetrahydrochloride, Sigma, St. Louis, MO) as a chromogen and counter-staining was performed with hematoxylin.

Co-localization of seprase and DPPIV was seen in microvessel endothelial-epithelial cells at the invasion front of human malignant breast ductal carcinoma. Human malignant breast ductal carcinomas were formaldehyde-fixed and paraffin embedded. Serial sections were stained with rat mAb D8 or D28 directed against seprase (labeled as Sep); rat mAb E26 or E19 directed against DPPIV (DPP4); mouse mAb against endothelial marker antigen CD34 or CD31 (Endo); or mouse mAbs against epithelial marker antigens C11, ESA and EMA (Epi). Open arrowheads indicate sites of micro-vessels located in the front of infiltrating sheets of poorly differentiated (high-grade) carcinoma that are identified as a putative "angiogenic center". In the center, the vessel lining cells were stained positively with seprase, DPPIV, endothelial and epithelial markers, and they are referred as "metastatic cells". Arrows indicate the carcinoma cells in tumor, and also a scale of 50 μm. Note that, in adjacent areas, seprase and DPPIV are differentially expressed in carcinoma and endothelial cells.

Distribution of seprase and DPPIV in mammary glands adjacent to their respective malignant breast ductal carcinoma tumors was assayed immunohistochemically. The tissues adjacent to malignant breast ductal carcinomas were formaldehyde-fixed and paraffin embedded side-by-side with the tumors. Serial sections were stained with rat mAb D8 or D28 directed against seprase; rat mAb E26 or E19 directed against DPPIV; mouse mAb against endothelial marker antigen CD34 or CD31; or mouse mAbs against epithelial marker antigens C11, ESA and EMA. Sites of micro-vessels were located surrounding the mammary glands. In most areas,

seprase and DPPIV were not detectable in all cell types, except that low levels of DPPIV were detected in a few mammary glands using high titer of primary antibodies, 10 μg/ml. Some background brown staining was seen on connective tissue fibers due to the high titer of the antibodies used.

As described above, protease expression was characterized in selected human breast carcinoma cell lines as well as in an immunohistochemistry (IHC) study using 70 cases of invasive ductal carcinoma and their respective, adjacent "normal" tissues. Seprase and DPPIV are expressed in a subset of carcinoma cells at the invasion front of tumor and in a subset of carcinoma cells at sites of putative "angiogenic center", but not other tumor cells and not in most epithelial and endothelial cells in adjacent tumor tissue. An intense but limited protease expression pattern was observed at putative "angiogenic sites" in the invasion front of tumors. At these sites, the carcinoma cells with large nuclei line the microvessel, express both seprase/DPPIV and epithelial/endothelial marker antigens, suggesting that these cells are of carcinoma origin and are invasive (based on their expression of seprase and DPPIV). However, both epithelial and endothelial cells in normal tissues adjacent to their respective tumors did not express seprase and DPPIV, except a subset of mammary epithelial cells had weak DPPIV staining.

These results described herein show that DPPIV of the seprase-DPPIV complex is selectively expressed in specific tissue types, namely granulated, metastatic tissues and other tissues in which angiogenesis and cell invasion are occurring and not normal tissue where the formation of new blood vessels and cell invasion have stopped. These tissues therefore provide an ideal target for therapeutic methods according to the present invention.

Example 18: Suppression of cancer invasion and metastasis with DPPIV antagonists as measured by *in vivo* experimental models. Monoclonal antibodies E19 and E26 that show positive effects in cell invasion and angiogenesis assays in culture were assessed for their in vivo activity using an experimental human invasion/metastasis model (Thompson et al., 1992). Human breast carcinoma cell line MDA-MB-436 (seprase+/DPPIV+) and human malignant melanoma cell line LOX

(seprase+/DPPIV-) were used as a basis for these animal studies. Seprase+/DPPIV+ and seprase+/DPPIV- cells were transformed with a retrovirus vector for lacZ tag as described (Kern et al., 1994) and these cells, $0.5x10^6$, were subcutaneously injected into 6-8 week-old female athymic (nu/nu) mice. Antibodies or inhibitors were subcutaneously co-inoculated orthotopically with human cancer cells (seprase+/DPPIV+ and seprase+/DPPIV-), followed by intravenous injection into the tail vein with 250 μg of the mAb E19, E26, or E3 (anti-DPPIV) twice a week for 2 to 3 weeks, to examine their effects on tumor invasion and tissue colonization as described (Kern et al., 1994). Injected mice were examined twice weekly for tumor sizes. Mice were maintained for 2-3 months or until primary tumor reaching 2cm in diameter, after which the primary tumor and selected organs (lung and liver) were assayed for β-galactosidase activity, histological analysis and immunohistochemical localization of endothelial cell type markers, seprase and DPPIV. Antibodies and inhibitors for MMPs, such as TIMP-2 or synthetic compounds CT1817 and Marimastat AG3340, as a positive inhibitory control for MMP effect, were used to test for the function of membrane proteases in vivo.

Tumors formed in nude mouse skin, which had been injected with human cancer cells (seprase+/DPPIV+ and seprase+/DPPIV-) were detectable and measureable. Morphological examination of the established tumors and lung metastases revealed that invasion and metastasis of human cancer cells into mouse tissue had occurred. The invasion and metastasis of human carcinoma cells were determined by the localization of blue-stained β-galactosidase tagged cancer cells and immunohistochemical staining using human-specific epithelial cell markers, including anti-human Muc-1 and epithelial surface antigen antibodies that were conjugated with a marker biotin. In addition, the blocking of the DPPIV by the intravenous application of DPPIV-specific E19 or E26 monoclonal antibodies resulted in a slowing of cancer invasion and lung metastasis in this model system.

Example 18: *In vivo* regression of human tumor growth and angiogenesis with DPPIV antagonists as measured by a chimeric m use-human assay. The effects of monoclonal antibodies on human tumor growth and angiogenesis were assessed by

co-inoculating human breast carcinoma cell line MDA-MB-436 (seprase+/DPPIV+) or human malignant melanoma cell line LOX (seprase+/DPPIV-) or ovarian cancer cells (seprase+/DPPIV+) with endothelial cells (CD31+) isolated from ovarian cancer ascites into the skin of SCID or nude mice. Severe combined immunodeficiency (SCID) mice, C.B-17-scid strain, were used in this assay. SCID lack both T and B cells due to a defect in V(D)J recombination, and SCID mice do not mount an antibody response to challenge by immunogenic material. Therefore, they easily accept foreign tissue transplants, including human cells. The SCID-human model was prepared essentially as described in (Yan et al., 1993). Briefly, seprase+/DPPIV+ and seprase+/DPPIV- cells tagged with lacZ were mixed with CD31+ human endothelial cells in Matrigel ($2x10^6$ cells each cell type), in the presence of monoclonal antibodies or inhibitors, were subcutaneously injected into 6-8 week-old SCID mice. Mice were housed in individual cages. The mice were then observed for 2 to 4 weeks to allow growth of measurable human tumors. Following the growth of measurable tumors, SCID mice were injected intravenously into the tail vein with 250 μg of either the mAb E19, E29 or E3 (anti-DPPIV) twice a week for 2 to 3 weeks. After this time, the tumors were resected from the skin and trimmed free of surrounding tissue. Three to four mice were used for each treatment and the result of suppression of tumor growth (as determined by size) with DPPIV antagonist antibodies are shown in Table I. The color of the tumor and immunohistochemistry results were used to indicate the degree of tumor angiogenesis. In this model, human tumor growth and neovascularization were occurring in the mouse tissue. The cell origin of the neovascularization within the human tumor was demonstrated by immunohistochemical staining of the neovasculature with human-specific endothelial cell markers, including anti-human factor VIII and CD31 antibodies.

TABLE I.

MDA-MB-436 Tumor Number	Treatment	Tumor Weights (mg)	Average Tumor Weights (mg)
1	E3/control	225	210
2	E3/control	203	
3	E3/control	179	
4	E3/control	213	
5	E19	125	102
6	E19	97	
7	E19	113	
8	E19	78	
9	E26	69	92
10	E26	89	
11	E26	134	
12	E26	77	

Exposure of the human carcinoma tumor mass in the mouse:human chimeric assay system to mabs E19 or E26 (anti-DPPIV antibodies that block cell migration and invasion in culture) caused the decrease from the E3 (a control, non-inhibitory anti-DPPIV antibody) treated average tumor weight of 210 mg to 102 mg and 92 mg, respectively. Representative examples of tumors treated with the mAb E26 (inhibitory anti-DPPIV) and E3 (control anti-DPPIV) were examined morphologically. The E3-treated tumors were large (11 to 14 mm in diameter) and well vascularized whereas those treated with mAb E26 (inhibitory anti-DPPIV) were much smaller (4 to 7 mm in diameter) and lacked detectable blood vessels. Immunohistochemical examination of the established tumors revealed that human neovascularization into the human tumor had occurred in mouse skin. The DPPIV

membrane protease was blocked by the intravenous application of inhibitory DPPIV E19 or E26 antibodies resulting in a regression of a human tumor in this model system in the same manner as the experimental invasion and metastasis model systems as described in the Examples above.

A therapeutically effective amount of DPPIV antagonist of this invention in the form of a monoclonal antibody, or fragment thereof, is typically an amount such that when administered in a physiologically tolerable composition is sufficient to achieve a plasma concentration of from about 0.01 microgram (μg) per milliliter (ml) to about 100 μg/ml, preferably from about 1 μg/ml to about 5 μg/ml, and usually about 5 μg/ml. Stated differently, the dosage can vary from about 0.1 mg/kg to about 300 mg/kg, preferably from about 0.2 mg/kg to about 200 mg/kg, most preferably from about 0.5 mg/kg to about 20 mg/kg, in one or more dose administrations daily, for one or several days.

The monoclonal antibodies of the invention can be administered by injection or by gradual infusion over time. Although the tissue to be treated can typically be accessed in the body by systemic administration and therefore most often treated by intravenous administration of therapeutic compositions, other tissues and delivery means are contemplated where there is a likelihood that the tissue targeted contains the target molecule. Thus, monoclonal antibodies or polypeptides of the invention can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, transdermally, and can be delivered by peristaltic means.

The therapeutic compositions containing a monoclonal antibody or a polypeptide of this invention are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluents; i.e., carrier, or vehicle. The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient,

whether the therapeutic composition is metabolized and/or excreted, and the degree of therapeutic effect desired.

Precise amounts of active ingredient to be administered may be determined by the practitioner by routine methods and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the route of administration. Suitable regimens for administration are also variable, but are typified by an initial dose followed by repeated doses at one or more hourly intervals by a subsequent injection or other routes of administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for *in vivo* therapies are contemplated.

As demonstrated by the present Examples, inhibition of angiogenesis and tumor regression occurs as early as 7 days after the initial contacting with antagonist. Additional or prolonged exposure to antagonist is preferable for 7 days to 6 weeks, preferably about 14 to 28 days.

Those of skill in the art will immediately recognize further embodiments of the present invention which are exemplified in the specification and claims herein presented.

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Claims:

1. A monospecific antibody which specifically binds an epitope of a mammalian DPPIV (dipeptidyl peptidase IV, also known as CD26).
2. The monospecific antibody of claim 1, wherein the antibody inhibits angiogenesis.
3. The monospecific antibody of claim 1, wherein the antibody is a monoclonal antibody or a polyclonal antibody.
4. The monoclonal antibody of claim 3, wherein the monoclonal antibody is an IgG.2a.
5. The monospecific antibody of claim 1, wherein the mammalian DPPIV (dipeptidyl peptidase IV/CD26) is human DPPIV.
6. The monospecific antibody of claim 2, wherein the antibody specifically binds the epitope bound by monoclonal antibody E19 or monoclonal antibody E26.
7. The monospecific antibody of claim 6, wherein the antibody is monoclonal antibody E19 or monoclonal antibody E26.
8. The monospecific antibody of claim 6, wherein the antibody comprises an antigen-binding fragment of monoclonal antibody E19 or of monoclonal antibody E26.
9. The monospecific antibody of claim 8, wherein the antigen-binding fragment is selected from the group consisting of $F(ab')_2$, F(ab') and Fv.
10. A bispecific antibody with binding specificity for a first epitope and a second epitope, wherein the first epitope is the epitope bound by the monospecific antibody of claim 1.
11. The bispecific antibody of claim 10, wherein the second epitope is an epitope of seprase, an epitope of MT1-MMP, an epitope of MMP-2 or an epitope of $\alpha(3)\beta(1)$-integrin.
12. The monospecific antibody of claim 1, wherein the antibody is a chimeric antibody.
13. The chimeric antibody of claim 12, wherein the antibody is a humanized antibody.
14. An immunoconjugate comprising a monospecific antibody according to claim

1 joined to a therapeutic agent.

15. The immunoconjugate of claim 14, wherein the antibody is a monoclonal antibody.

16. The immunoconjugate of claim 15, wherein the monoclonal antibody is an IgG.2a.

17. The immunoconjugate of claim 15, wherein the immunoconjugate specifically binds the epitope bound by monoclonal antibody E19 or monoclonal antibody E26.

18. The immunoconjugate of claim 17, wherein the immunoconjugate comprises monoclonal antibody E19 or monoclonal antibody E26.

19. The immunoconjugate of claim 17, wherein the immunoconjugate comprises an antigen binding fragment of monoclonal antibody E19 or of monoclonal antibody E26.

20. The immunoconjugate of claim 19, wherein the antigen binding fragment is selected from the group consisting of $F(ab')_2$, F(ab') and Fv.

21. The immunoconjugate of claim 14, wherein the immunoconjugate comprises a humanized antibody.

22. The immunoconjugate of claim 14, wherein the immunoconjugate comprises a single chain antibody.

23. An immunoconjugate comprising a bispecific antibody with binding specificity for a first and a second epitope, the first epitope being an epitope of human DPPIV (dipeptidyl peptidase IV/CD26).

24. The immunoconjugate of claim 23, wherein the second epitope is an epitope of human seprase, MT1-MMP, MMP-2 or $\alpha(3)\beta(1)$-integrin.

25. The immunoconjugate of claim 14, wherein the therapeutic agent is an anti-tumor drug, a cytotoxin, a radioactive agent, a photosensitizer, a second antibody or an enzyme.

26. A pharmaceutical composition for inhibiting angiogenesis comprising an effective amount of a monospecific antibody according to claim 2 and a pharmaceutically acceptable carrier.

27. A pharmaceutical composition for inhibiting angiogenesis comprising an effective amount of a bispecific antibody according to claim 10 and a

pharmaceutically acceptable carrier.

28. A pharmaceutical composition for inhibiting angiogenesis comprising an effective amount of an immunoconjugate according to claim 14 and a pharmaceutically acceptable carrier.

29. A pharmaceutical composition for inhibiting angiogenesis comprising an effective amount of an immunoconjugate according to claim 23 and a pharmaceutically acceptable carrier.

30. A method of treating a patient suffering from a growth or proliferative disorder involving angiogenesis, comprising administering an effective amount of a monospecific antibody according to claim 2.

31. A method of treating a patient suffering from a growth or proliferative disorder involving angiogenesis, comprising a administering an effective amount of a bispecific antibody according to claim 10.

32. A method of treating a patient suffering from a growth or proliferative disorder involving angiogenesis, comprising administering an effective amount of an immunoconjugate according to claim 14.

33. A method of treating a patient suffering from a growth or proliferative disorder involving angiogenesis, comprising administering an effective amount of an immunoconjugate according to claim 23.

34. The method of treating a patient according to claim 30, in combination with a chemotherapy regimen.

35. The method of treating a patient according to claim 31, in combination with a chemotherapy regimen.

36. The method of treating a patient according to claim 32, in combination with a chemotherapy regimen.

37. The method of treating a patient according to claim 33, in combination with a chemotherapy regimen.

38. A continuous cell line which produces the monospecific antibody of claim 1.

39. The continuous cell line of claim 38, wherein the monospecific antibody is a monoclonal antibody.

40. The continuous cell line of claim 39, wherein the monoclonal antibody specifically binds the epitope recognized by monoclonal antibody E19 or

monoclonal antibody E26.

41. The continuous cell line of claim 39, wherein the monoclonal antibody specifically binds the epitope recognized by monoclonal antibody E3 or monoclonal antibody F4.

42. The continuous cell line of claim 40, wherein the monoclonal antibody is E19.

43. The continuous cell line of claim 40, wherein the monoclonal antibody is E26.

44. The continuous cell line of claim 42, which is the E19 hybridoma.

45. The continuous cell line of claim 43, which is the E26 hybridoma.

46. A method of inhibiting cancer invasion and angiogenesis in a solid tumor in a patient wherein cells of normal tissue do not express levels of the DPPIV-seprase complex detectable by immunohistochemistry, the method comprising administering to said patient a composition comprising an cancer invasion- and angiogenesis-inhibiting amount of anti-DPPIV monoclonal antibody, whereby the DPPIV-seprase complex expressed on the surface of vascular endothelial cells and invading cancer cells involved in said cancer invasion and angiogenesis is contacted by said antibody resulting in inhibition of cancer invasion and limiting the blood supply to the tissue of said solid tumor.

47. The method of claim 46, wherein said anti-DPPIV antibodies inhibit binding of collagen to the DPPIV-seprase complex.

48. The method of claim 46, wherein said monoclonal antibody has the immunoreactivity characteristics of monoclonal antibody E19 or monoclonal antibody E26.

49. The method of claim 46, wherein the cancer invasion and angiogenesis-inhibiting amount of anti-DPPIV monoclonal antibody is from about 0.1 mg/kg to about 300 mg/kg.

50. The method of claim 46, wherein said administering comprises intravenous administration.

51. The method of claim 46, wherein said administering comprises transdermal administration.

52. The method of claim 46, wherein said administering comprises intramuscular administration.

53. The method of claim 46, wherein said administering comprises oral

administration.

54. The method of claim 46, wherein said administering is conducted in conjunction with chemotherapy.

55. The methods of claim 46, wherein said administering is conducted in conjunction with administration of a cytotoxin conjugate.

56. The method of claim 46, wherein the patient is a human.

57. The method of claim 56, wherein the antibody is humanized.

58. The method of claim 57, wherein the humanized antibody has the immunoreactivity characteristics of monoclonal antibodies E19 and E26.

59. The method of claim 46, wherein the anti-DPPIV antibody specifically binds the DPPIV-seprase complex.

60. The method of claim 46, wherein the tumor is metastasized.

61. A method of stimulating angiogenesis in a mammal mammal suffering from disease or disorder that may be remedied by an increased blood supply, the method comprising administering to said mammal a composition comprising an angiogenesis-stimulating amount of a DDPIV modulator, whereby the blood supply to the affected tissue is increased.

62. The method according to claim 61, wherein the disease or disorder is a cardiovascular disease, a diabetic ulcer, a retinopathy or a non-healing wound.

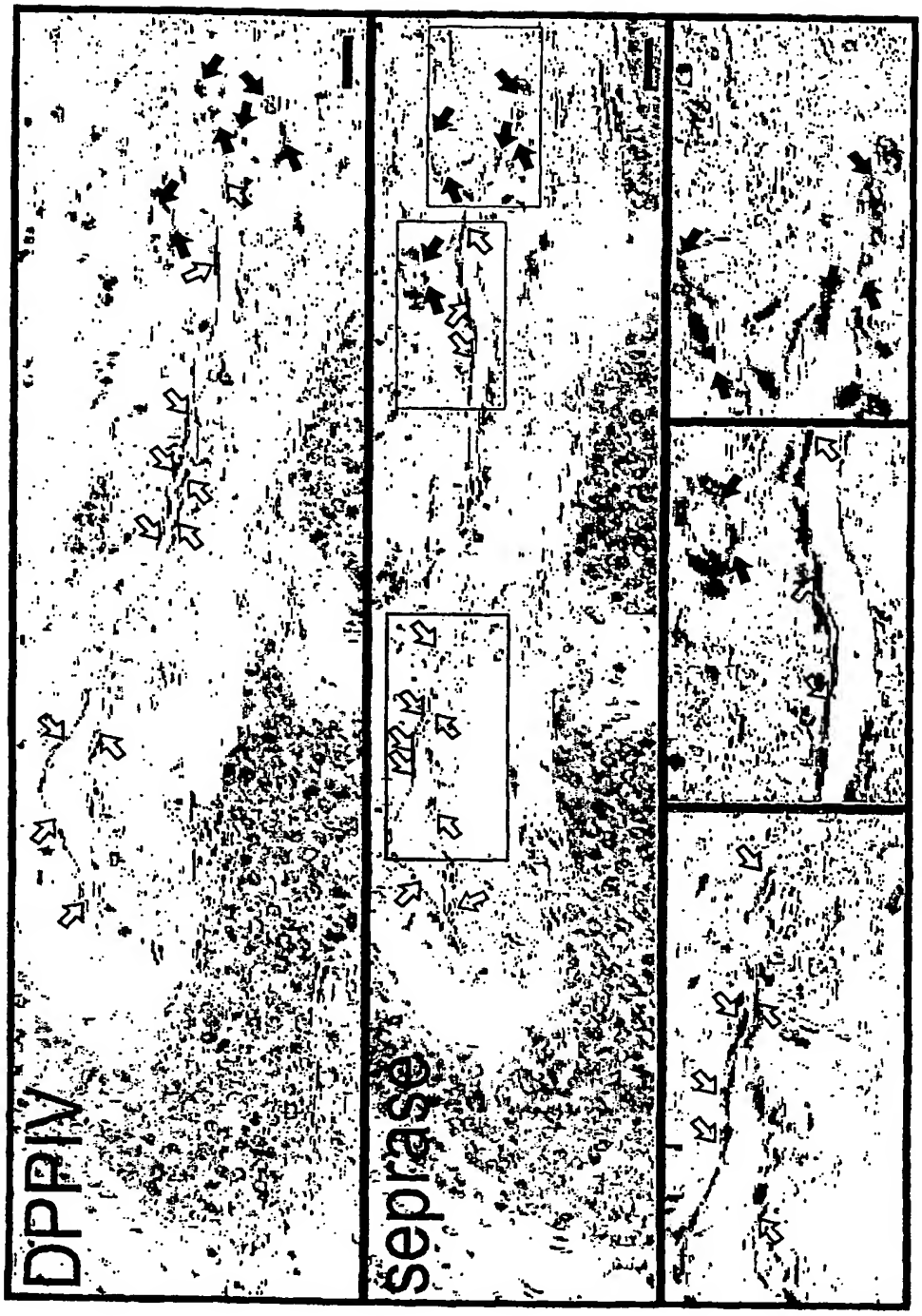


FIG. 1

FIG. 2A

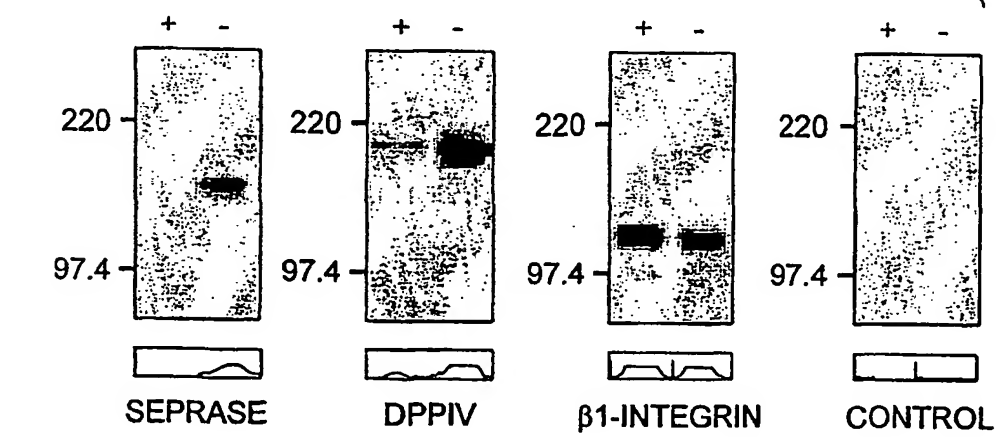


FIG. 2B

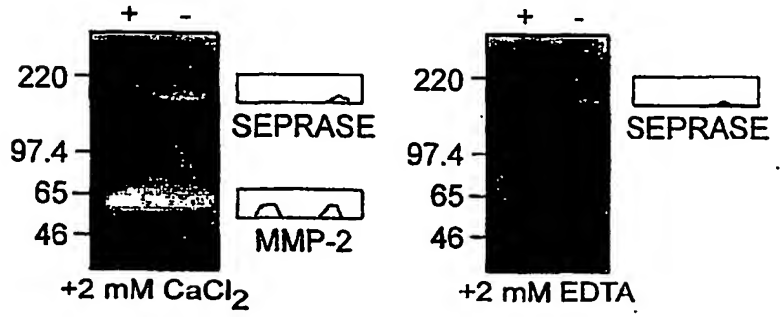


FIG. 2C

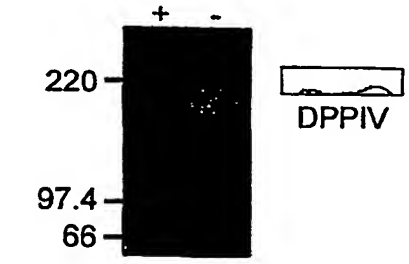


FIG. 2D

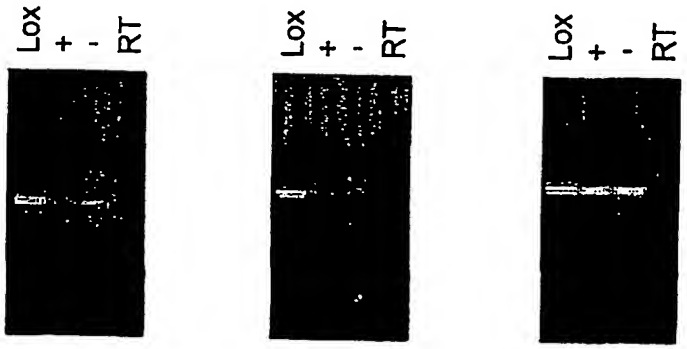


FIG. 2E

FIG. 2F

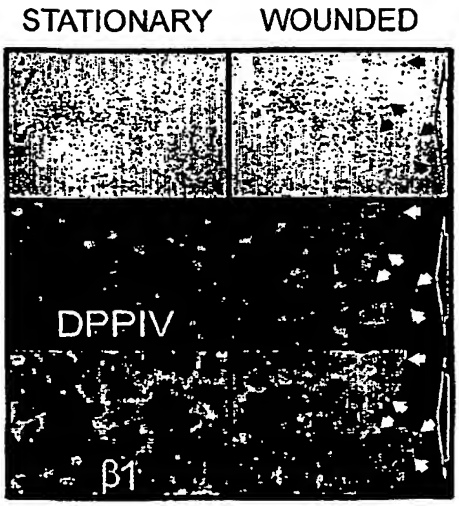
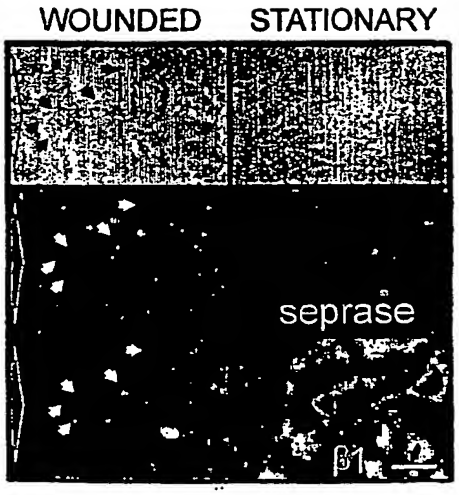


FIG. 2G



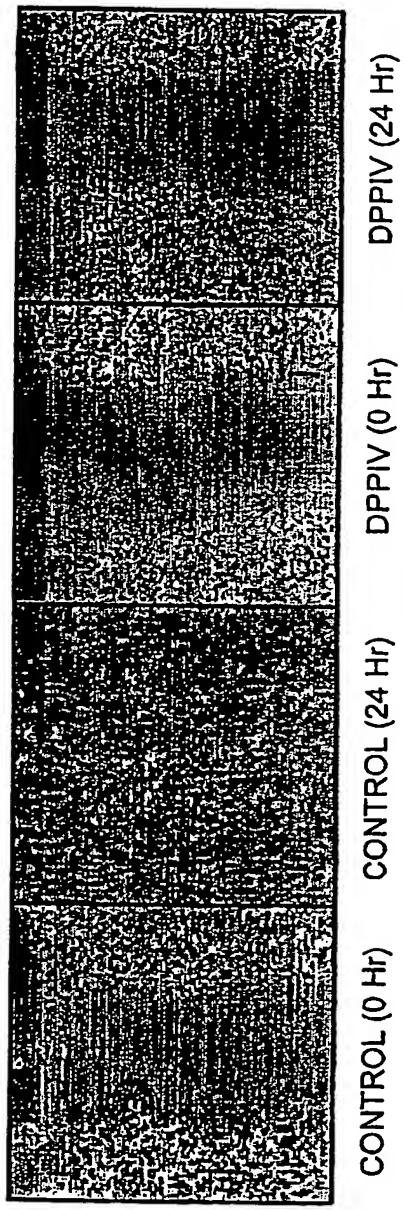


FIG. 2H

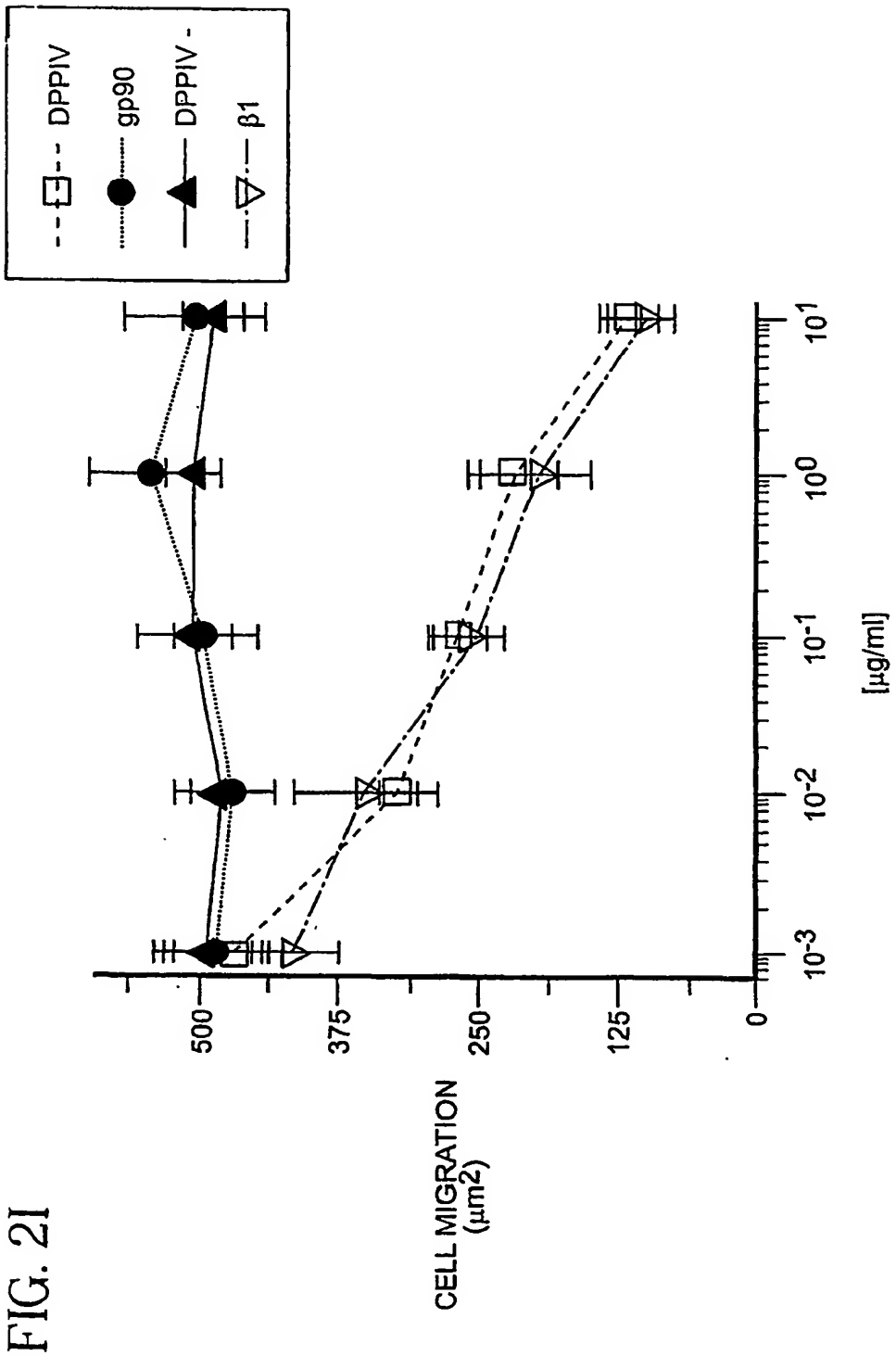

DPPIV
gp90
DPPIV -
β1
CELL MIGRATION (μm2)
500
375
250
125
0
10-3
10-2
10-1
100
101
[μg/ml]

FIG. 21

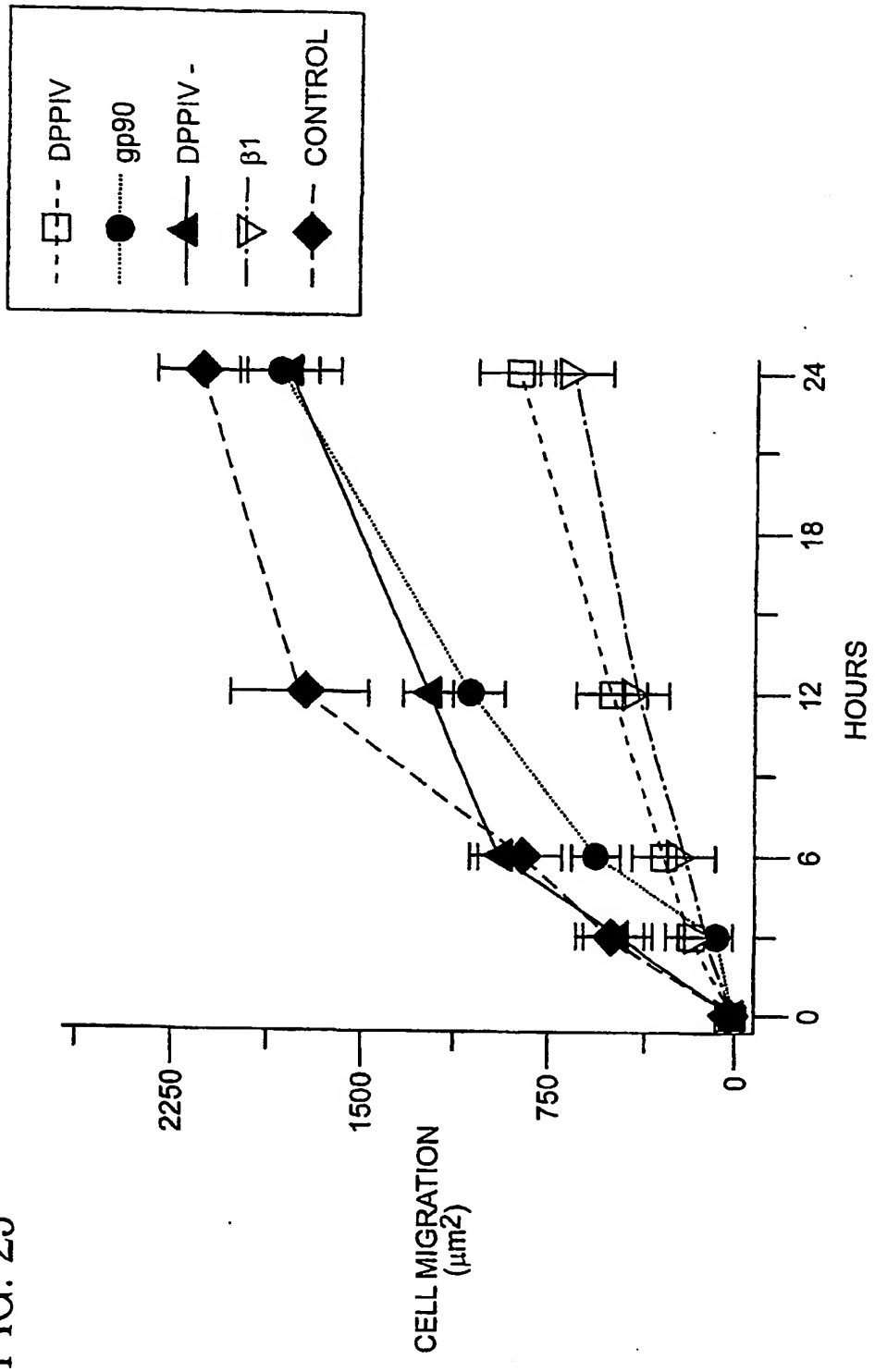


FIG. 2J

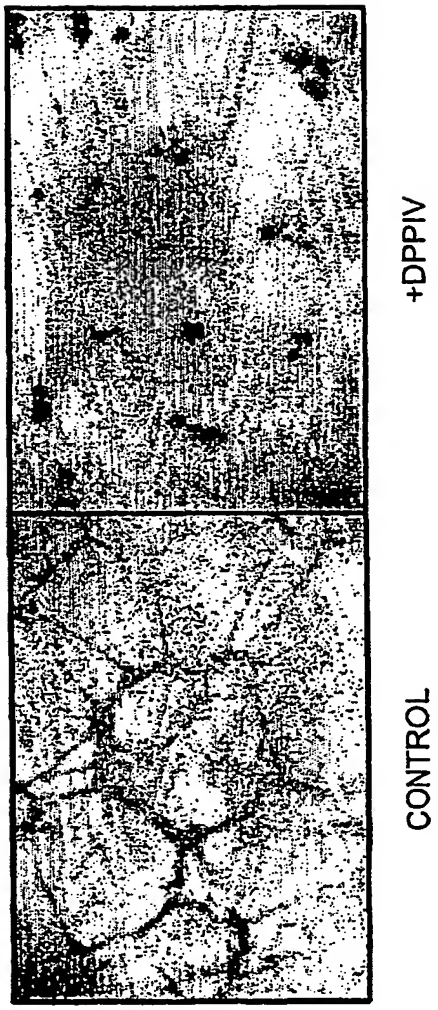


FIG. 3A

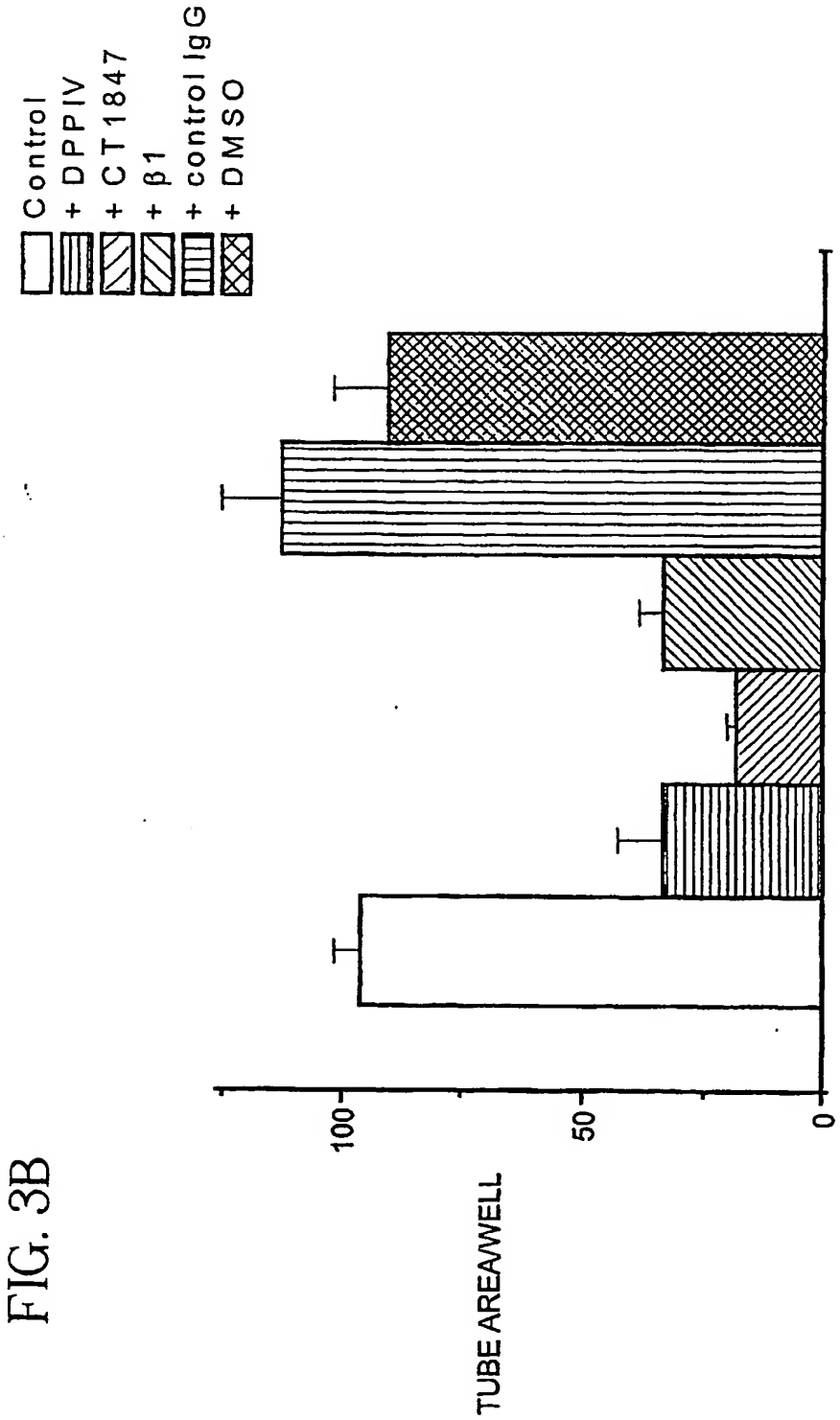


FIG. 3B

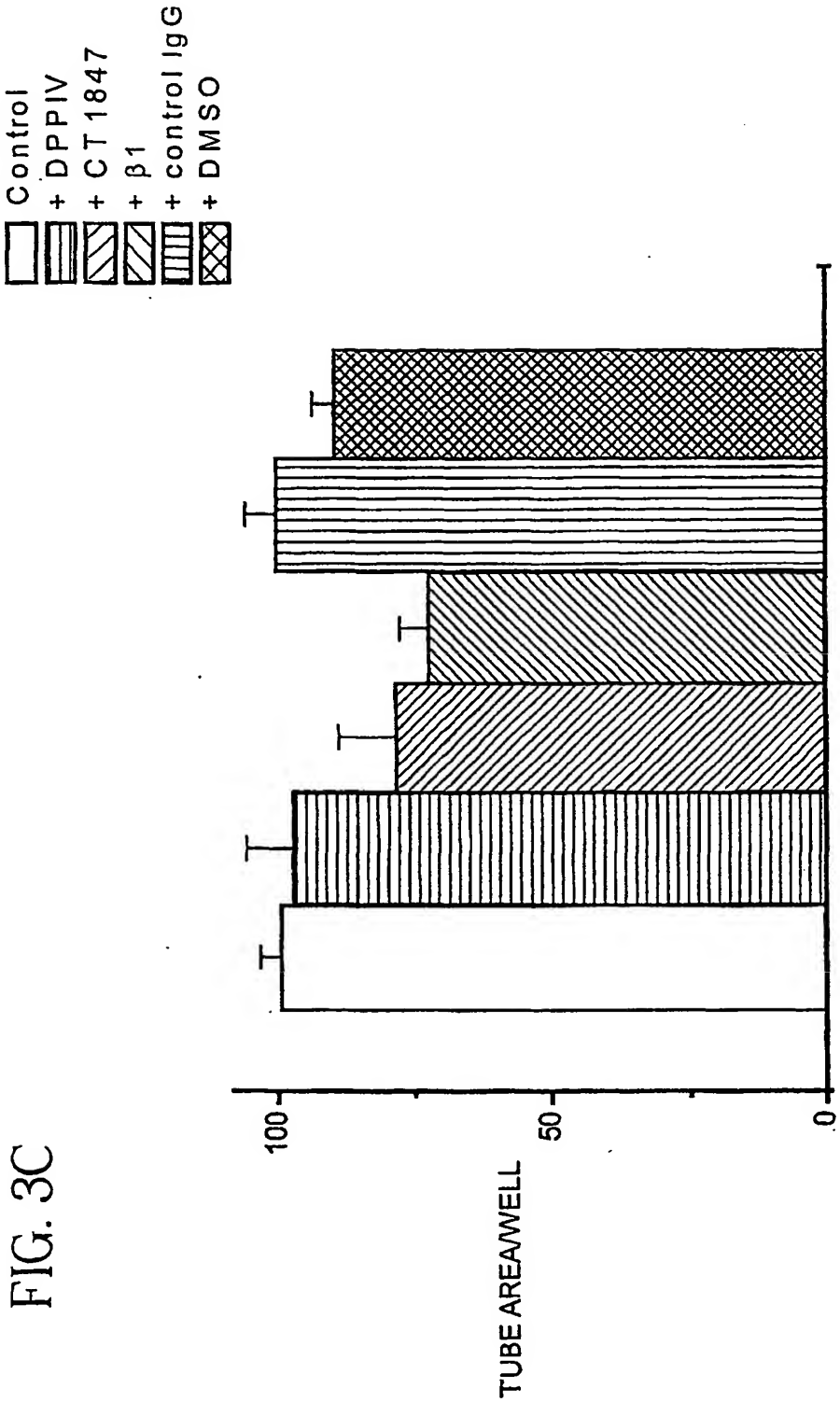

Control
+ DPPIV
+ CT 1847
+ β1
+ control IgG
+ DMSO
100
50
0
TUBE AREA/WELL

FIG. 3C

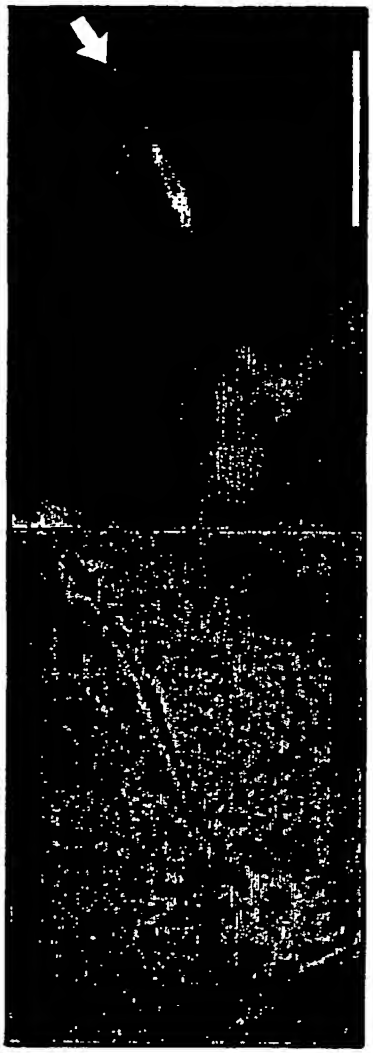


FIG. 3D

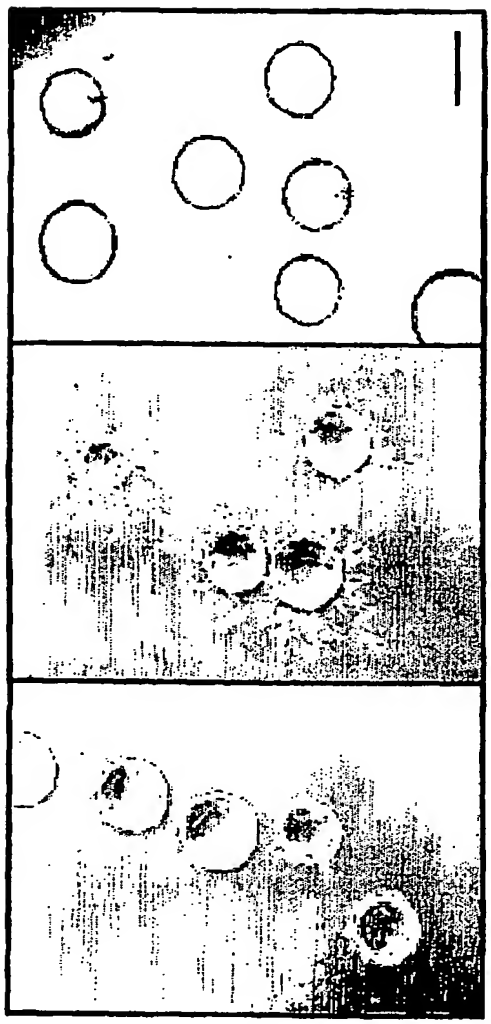


FIG. 4A

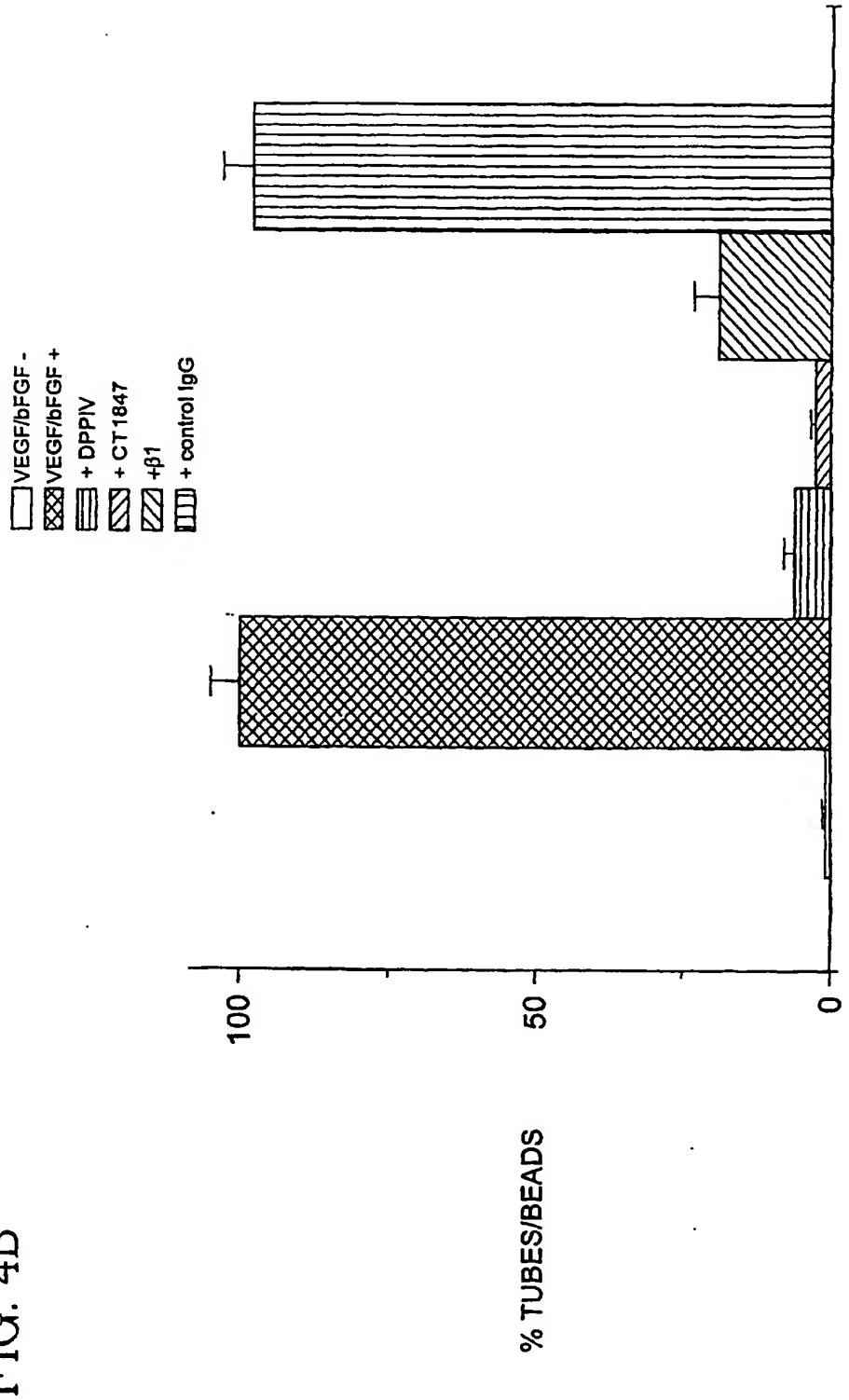


FIG. 4B

FIG. 5

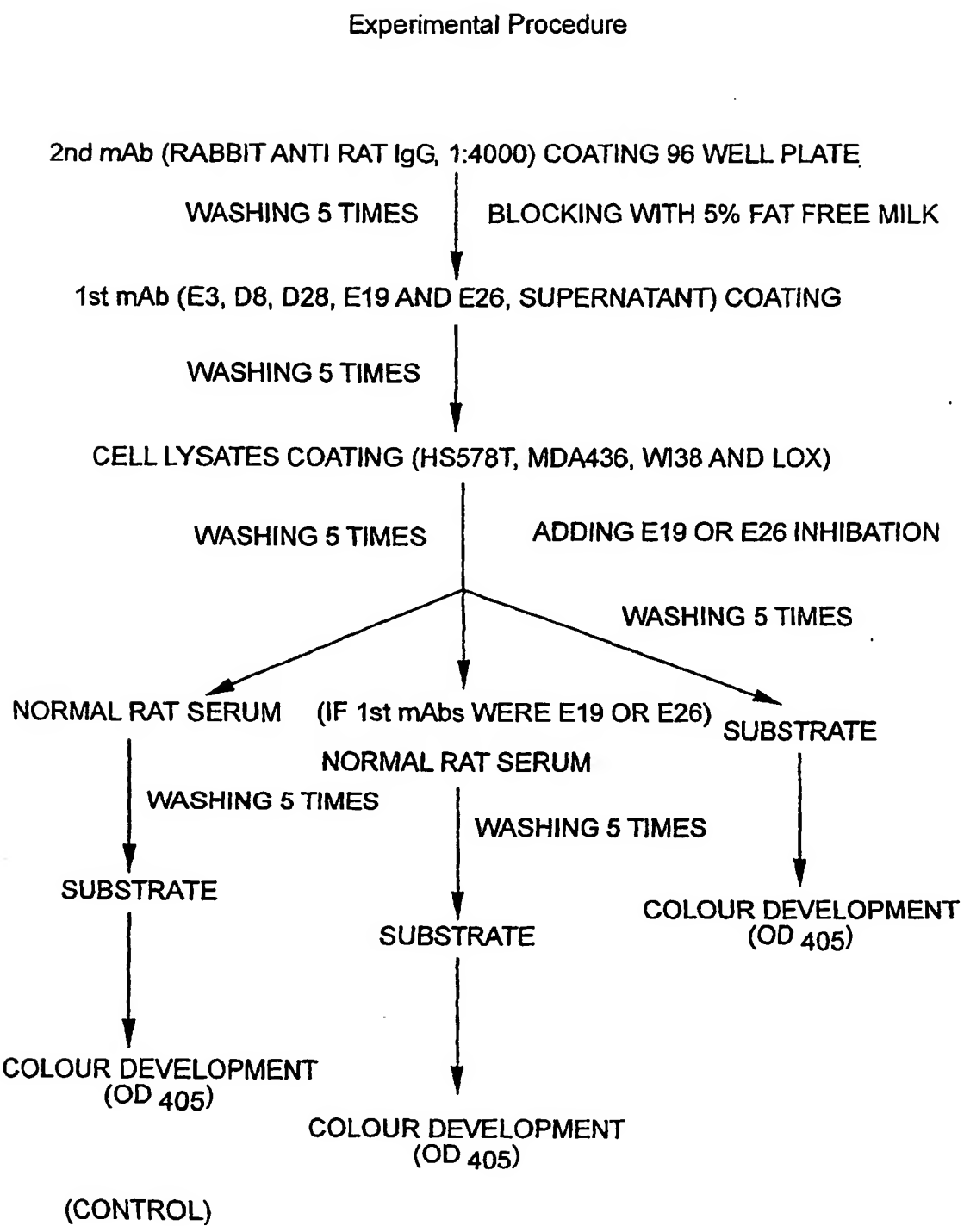


FIG. 6A

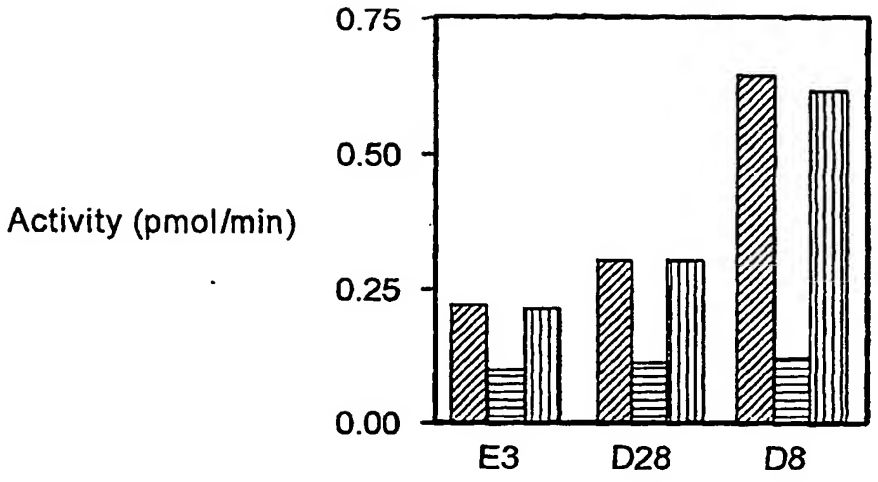


FIG. 6B

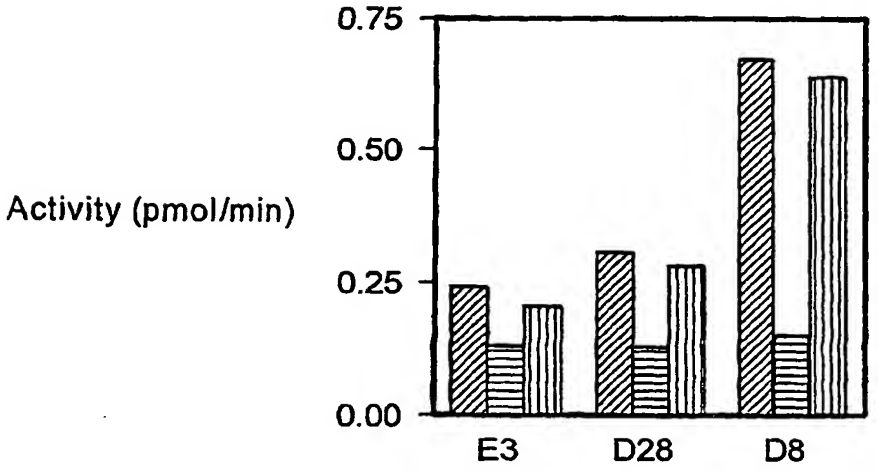


FIG. 6C

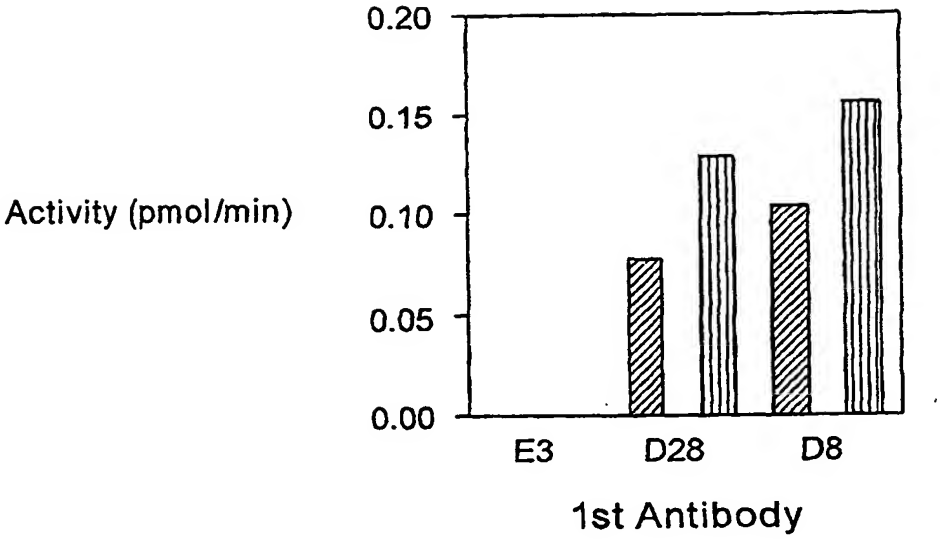

Activity (pmol/min)
0.20
0.15
0.10
0.05
0.00
E3
D28
D8
1st Antibody

FIG. 6D

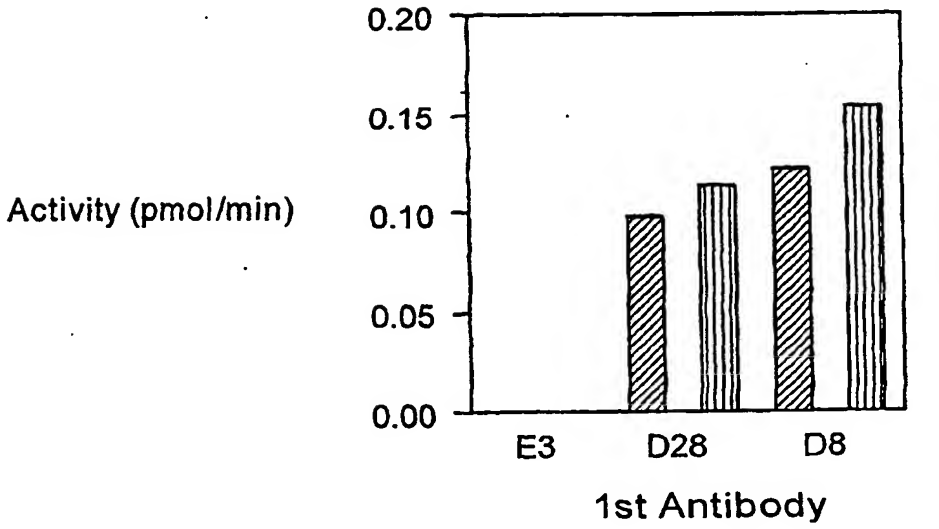

Activity (pmol/min)
0.20
0.15
0.10
0.05
0.00
E3
D28
D8
1st Antibody

FIG. 7A

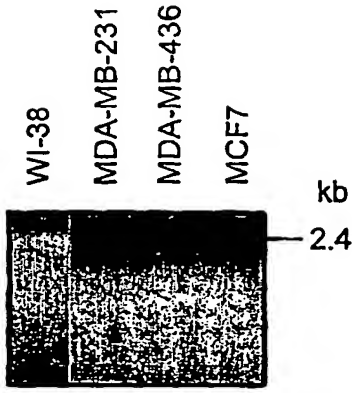


FIG. 7B

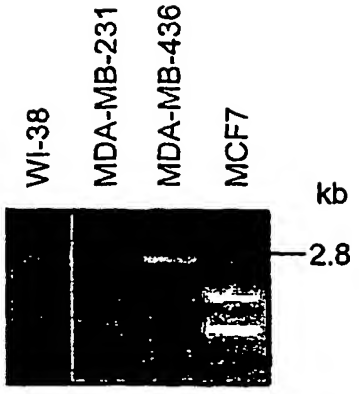


FIG. 7C

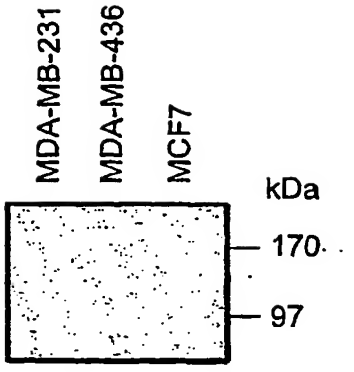


FIG. 7D

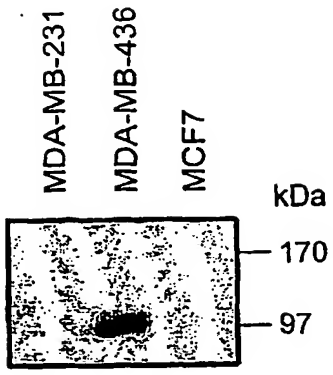


FIG. 7E

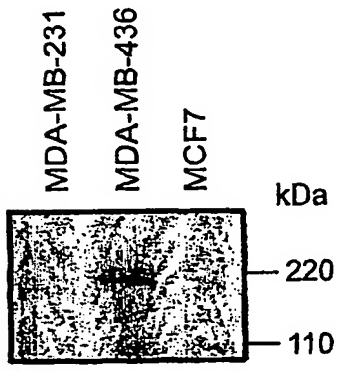


FIG. 7F

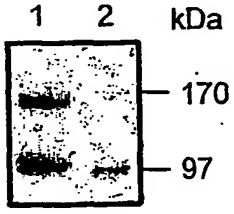


FIG. 8A

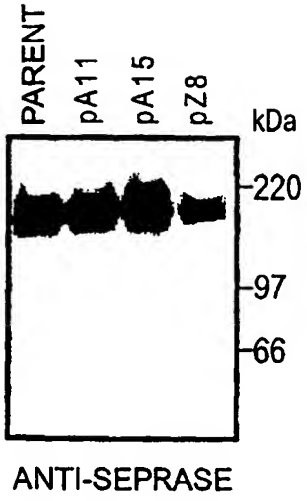

PARENT
pA11
pA15
pZ8
kDa
220
97
66
ANTI-SEPRASE

FIG. 8B

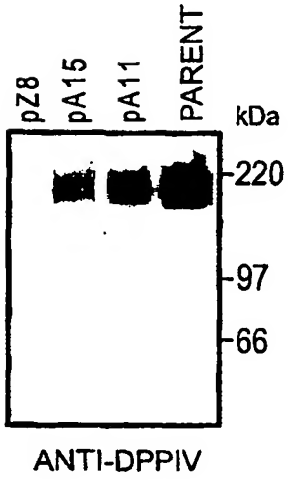

pZ8
pA15
pA11
PARENT
kDa
220
97
66
ANTI-DPPIV

FIG. 8C

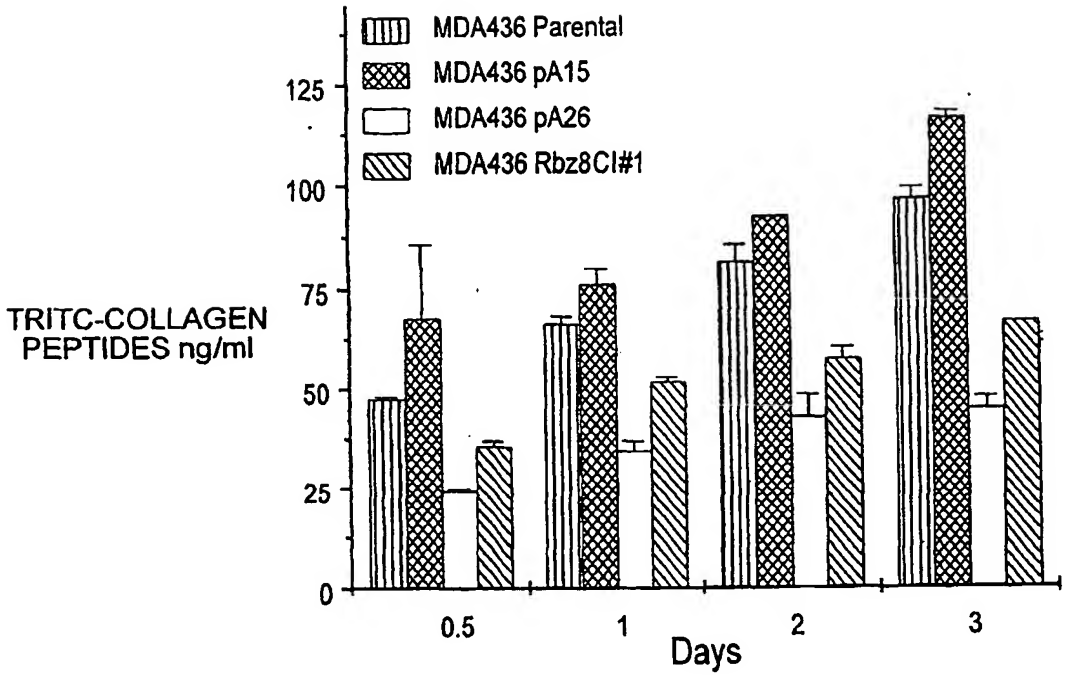

MDA436 Parental
MDA436 pA15
MDA436 pA26
MDA436 Rbz8CI#1
TRITC-COLLAGEN PEPTIDES ng/ml
125
100
75
50
25
0
0.5
1
2
3
Days

FIG. 9A

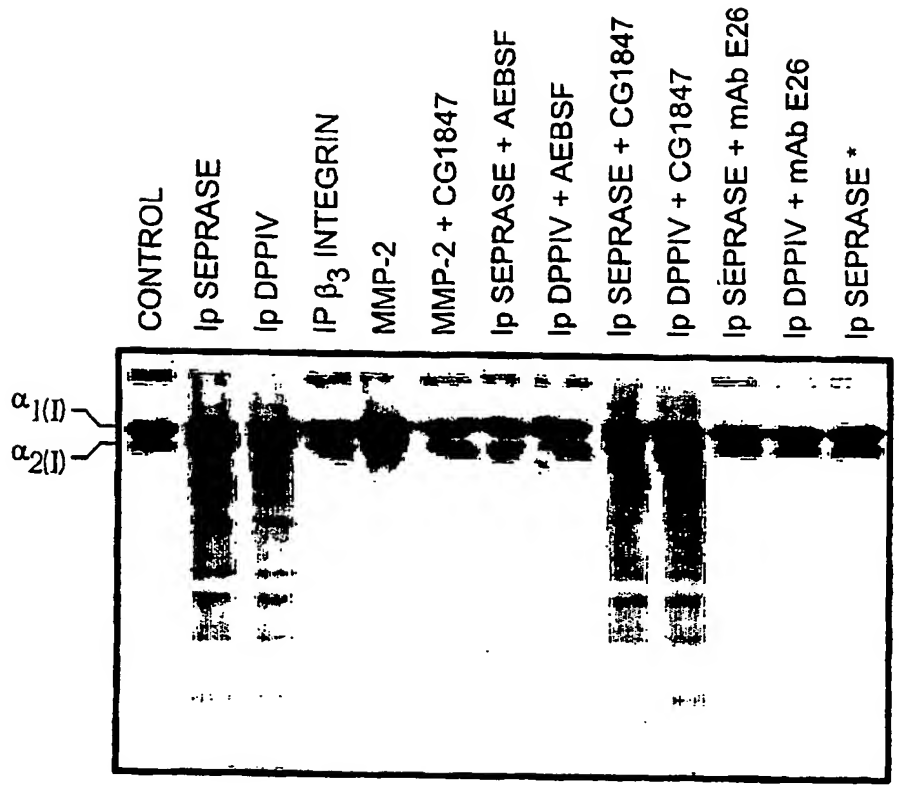
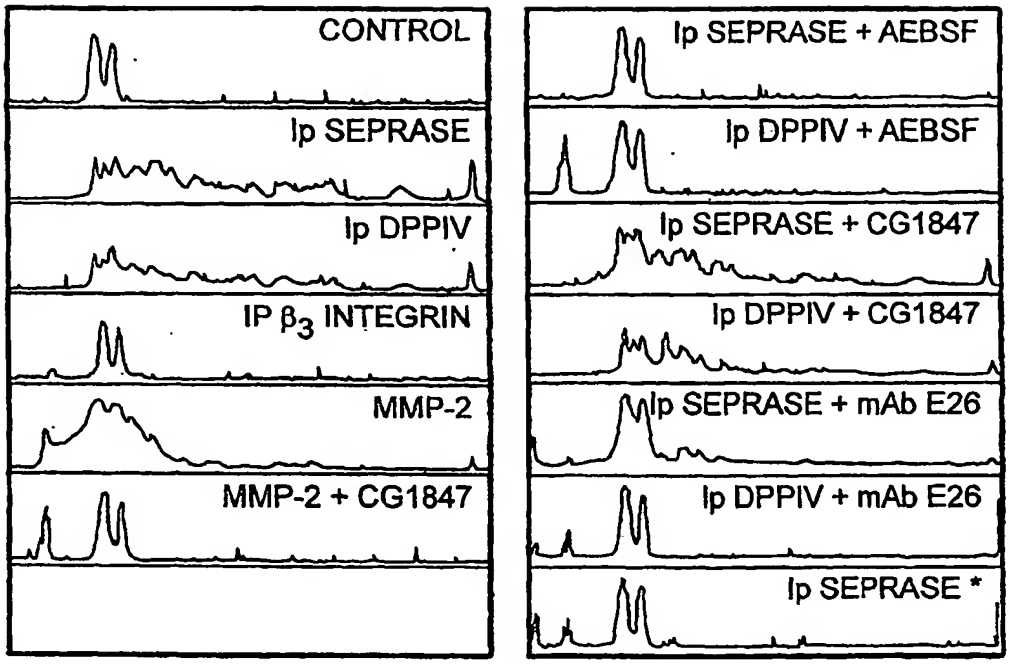

CONTROL
Ip SEPRASE
Ip DPPIV
IP β_3 INTEGRIN
MMP-2
MMP-2 + CG1847
Ip SEPRASE + AEBSF
Ip DPPIV + AEBSF
Ip SEPRASE + CG1847
Ip DPPIV + CG1847
Ip SEPRASE + mAb E26
Ip DPPIV + mAb E26
Ip SEPRASE *
α_1(I)
α_2(I)

FIG. 9B


CONTROL
Ip SEPRASE
Ip DPPIV
IP β_3 INTEGRIN
MMP-2
MMP-2 + CG1847
Ip SEPRASE + AEBSF
Ip DPPIV + AEBSF
Ip SEPRASE + CG1847
Ip DPPIV + CG1847
Ip SEPRASE + mAb E26
Ip DPPIV + mAb E26
Ip SEPRASE *

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(54) Title: METHOD AND COMPOSITIONS FOR ISOLATING METASTATIC CANCER CELLS, AND USE IN MEASURING METASTATIC POTENTIAL OF A CANCER THEREOF

(57) Abstract: The present invention relates to novel methods and compositions for detection and isolation of cancer cells with metastatic potential. The invention further relates to assays for measuring the metastatic potential of such cancer cells and drug screening assays for the identification of agents having anti-metastatic potential. The present invention further provides methods and compositions for inhibiting the metastatic potential of cancer cells by modulating the activity of serine integral membrane proteases [(SIMP) consisting of seprase and dipeptidyl peptidase IV (DPPIV)]expressed on the surface of metastasizing cancer cells.

METHODS AND COMPOSITIONS FOR ISOLATING METASTATIC CANCER CELLS, AND USE IN MEASURING METASTATIC POTENTIAL OF A CANCER THEREOF

SPECIFICATION

1. INTRODUCTION

The present invention relates to novel methods for detection and isolation of cancer cells with metastatic potential from blood, ascites and tumor tissue derived from subjects with metastatic cancer. The invention further relates to novel compositions for use as cell-adhesion matrices for detection and isolation of cancer cells and for use as blood filters by subjects having metastatic cancers. The methods and compositions of the invention may also be used in assays designed for measuring the metastatic potential of isolated cancer cells and for identification of agents having anti-metastatic potential. In addition, the invention relates to inhibiting the metastatic potential of cancer cells by modulating the activity of serine integral membrane proteases expressed on the surface of metastasizing cancer cells. The present invention is based on the discovery that carcinoma cells isolated from patients' blood, ascites or tumor preferentially adhere to, degrade and ingest collagenous matrix materials. Further, it was discovered that a serine integral membrane protease (SIMP) consisting of seprase and dipetidyl peptidase IV (DPPIV) subunits are activated on the surface of migrating cells.

2. BACKGROUND OF THE INVENTION

Problems associated with cancer cell separation from the ` or tissue of patients with metastatic cancer during traditional bone marrow harvest and leukopheresis procedures have been reported (Campana, D. et al. Detection of Minimal Residual Disease in Acute Leukemia: Methodologic Advances and Clinical Significance, *Blood,* 1995 Mar 15, 85(6): 1416-34; Brugger, W. et al., Mobilization of Tumor Cells and Hematopoietic Progenitor Cells into Peripheral Blood of Patients with Solid Tumors, *Blood*, 83(3): 636-40, 1994). It is estimated that among the order of 10 billion total mononuclear cells harvested from a patient, there are 25 thousand to

12 million contaminating cancer cells. These contaminating cancer cells have been shown by genetic marking to contribute to relapse (Rill, E R et al., Direct Demonstration that Autologous Bone Marrow Transplantation for Solid Tumors Can Return a Multiplicity of Tumorigenic Cells, *Blood,* 84(2): 380-383, 1994).

Large numbers of cancer cells were also found in the circulation of cancer patients with metastatic diseases. Glaves, D., RP Huben, & L. Weiss (1988. Br. J. Cancer. 57:32-35) took samples of blood from the renal vein in 10 patients just prior to surgery of renal cell carcinoma and estimated that cancer cells were being released at rates of 10^7 to 10^9 cells per day. How these circulating cancer cells contribute to metastasis remains unknown. A major stumbling block is the difficulty involved in identifying an extremely minor subpopulation of circulating cancer cells, ranging from one of thousands to millions of cells, which are metastatic. It is apparent that majority of circulating cancer cells are killed due to host immunity. For examples, in experimental animal tumor models where the use of antibody-based cell separation is more reliable, it has been estimated that about 10 to 100 million tumor cells are released into the blood during the growth of transplantable B16 melanomas and Lewis lung tumors (approximately 20 days), however, these cells give rise to less than 100 lung metastases per mouse (Glaves, D., 1983, Br. J. Cancer, 48:665-673). Furthermore, in the large number of experiments in which tumor cells have been introduced directly into the circulation of mice or rats it is rare that more than 0.01% of such cells form tumor nodules. More commonly the efficiency is two or more orders of magnitude lower. These experimental data suggest that the initial release of cancer cells from the primary tumor is not the limiting factor in metastatic development as only a very small fraction of shed cancer cells are viable, invasive, and, therefore, metastatic. It is essential to develop a cell separation and detection system targeting on such metastatic cells for the understanding of mechanism of metastasis.

Several methods are known for separating cancer cells from blood or body fluids. Such methods include, for example, separating cancer cells one by one by microdissection (Suarez-Quian et al., 1999, *Biotechniques*, 26:328-35; Beltinger and Debatin, 1998, *Mol. Pathol* 51:233-6) or by antibody-based methods using

fluorescence activated cell sorting (Pituch-Noworolska et al., 1998, *Int. J. Mol. Med.* 1:573-8), separating cancer cells coated with antibodies on a magnetic material through the use of a magnetic field (Denis et al., 1997, *Int. J. Cancer* 74:540-4; Racila et al., 1998, *Proc. Natl. Acad Sci USA* 95-4589-94), or separating circulating cancer cells on density gradients (Sabile et al., 1999, *Am. J. Clin. Pathol.* 112:171-8). However, such cancer cell separation methods are dependent on the availability of tumor specific antibodies or the buoyant density and morphology unique to different cancer cells. Thus, a great need exists for efficient methods for removing cancer cells from a hematopoietic cell transplant (Gulati, S C et al. Rationale for Purging in Autologous Stem Cell Transplantation. *Journal of Hematotherapy*, 2(4):467-71, 1993).

As demonstrated in early studies, primary cancers begin shedding neoplastic cells into the circulation at an early stage of metastases formation (Fidler IJ, 1973, *European Journal of Cancer* 9:223-227; Liotta LA et al., 1974, *Cancer Research* 34:997-1004). Once shed into the circulation, cancer cells adhere to the basement membrane underlying vessel walls and invade adjacent connective tissue leading to formation of micrometastases (Liotta et al., 1991, *Cell* 64:327-336). It is postulated that cancer cells present in the invasion front and those shed into the circulation are critically involved in the progression of metastatic diseases.

The metastatic process is complex, involving escape of a cancer cell from the primary tumor, movement to a new location and establishment of growth at the new site. To successfully metastasize, the invasive cancer cells must acquire the following metastatic properties: (i) shedding from primary carcinoma, (ii) survival in the circulation and growth on vessel wall, (iii) the ability to invade (adhere to, and subsequently degrade and ingest) collagenous matrix, and (iv) extravasation, colonization and cooperation with angiogenesis (Chambers et al., 1998, *Cancer & Metastasis Review* 17:263-269).

The various steps associated with the process are essentially the same whether the cell escapes into lymphatic or blood vessels, and they involve an essential cellular property, *i.e.*, cell invasiveness. Cancer invasiveness requires the adhesion to, and the degradation and ingestion of the extracellular matrix (ECM) by invading

cancer cells, accompanied by translocation or migration of the cells into the ECM. Such cellular activities occur on membrane protrusions referred to as invadopodia, which exhibit dynamic membrane mobility, ECM adhesion, and degradation. Recent evidence has demonstrated the involvement of serine integral membrane proteases (SIMP), including dipetidyl peptidase IV (DPPIV)/CD26 and seprase, in cell surface proteolysis (Chen, W-T, 1996, *Enzyme Protein* 49:59-71).

SIMP members are type II transmembrane proteins comprising cytoplasmic tails that contain 6 amino acids followed by a 20 (seprase) or 22 (DPPIV) amino acid transmembrane domain at the N-terminus and a stretch of 200 amino acids at the C-terminus that constitutes a catalytic region with the catalytic serine in a non-classical orientation (Goldstein, LA et al., 1997, *Biochem. Biophys. Acta.* 1361:11-19). DPPIV specifically removes N-terminal dipeptides from oligo-peptides with either L-proline, L-hydroxyproline, or L-alanine at the penultimate position. Such peptides include Neuro-Peptide Y and other peptide hormones (Heins, J et al., 1988, *Biochim. Biophys. Acta* 954:161-169; Walter, R et al., 1980, *Mol. Cell Biochem.* 30:111-126). In addition, a recent report showed that DPPIV also possesses a seprase-like gelatinase and endopeptidase activity, suggesting its involvement in collagen degradation (Bermpohl F et al., 1998, *FEBS Letters* 428:152-156). In addition, DPPIV is expressed constitutively on brush border membranes of intestine and kidney epithelial cells (Yaron and Naider, F, 1993, *Crit. Rev. Biochem. Mol. Biol.* 28:31-81; Morimoto C. and Schlossman SF, 1994, *Immunologist* 2:4-7) and transiently expressed on T-cells implicating DPPVI as a marker for T-cell activation (Morimoto C. and Schlossman SF, 1994, *Immunologist* 2:4-7).

Seprase, originally identified as a 170-kDa membrane-bound gelatinase is expressed on invadopodia of highly aggressive melanoma LOX (Aoyama A. and Chen, W.T., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:8296-8300; Mueller, SC et al., 1999, J. Biol Chem. 274:24947-24952; Monsky, WL et al., 1994, *Cancer Res.* 54:5702-5710). The active enzyme was isolated from cell membranes and shed vesicles of LOX cells. Seprase is a homodimer of 97- kDa subunits (Pineiro-Sanchez, ML et al., 1997, *J. Biol. Chem.* 272:7595-7601). Analysis of the deduced amino acid sequence derived from a cDNA that encodes the 97-kDa subunit reveals that the 97-

kDa subunit is homologous to DPPIV, and is essentially identical to fibroblast activation protein α (FAPα) (Goldstein et al., 1997 *Biochem. Biophys. Acta.* 1361, 11-19; Scanlon, M.J et al., 1994, *Proc. Natl. Acad. Sci.* U.S.A., 91:5657-5661). FAP α is expressed on reactive stromal fibroblasts of epithelial cancers and in healing wounds but not in adult tissue (Garin-Chesa, P. et al., 1990, *Proc. Natl. Acad. Sci.* U.S.A. 87:7235-7239).

In carcinoma tumors, however, FAPα was not found to be expressed in carcinoma or endothelial cells (Garin-Chesa et al., 1990, *Proc. Natl. Acad. Sci.* 87:7235-7239). Seprase and FAPα differ mainly in a stretch of 45 amino acid residues contiguous with the highly conserved motif GXSXG that contains the active site serine (Scanlan et al., 1994, *Proc. Natl. Acad. Sci. USA* 91:5657-5661; Goldstein et al., 1997 *Biochem. Biophys. Acta.* 1361, 11-19). Recently, an alternatively spliced seprase mRNA was identified from the human melanoma cell line LOX that encodes a novel truncated 27-kDa seprase isoform, that precisely overlaps the carboxyl-terminal catalytic region of 97-kDa seprase (Goldstein and Chen, 2000 *J. Biol. Chem. 275:2554-2559*). The splice variant mRNA is generated by an out-of-frame deletion of a 1223-base pair exonic region that encodes part of the cytoplasmic tail, transmembrane, and the membrane proximal-central regions of the extracellular domain (Val(5) through Ser(412)) of the seprase 97-kDa subunit. It is possible that seprase exhibits both gelatinase and Gly-Pro-dipeptidase activities, while the truncated seprase only has the latter dipeptidase activity.

It has long been believed that collagen remodeling is mediated by matrix metalloproteinases (MMP). However, trials with MMP inhibitors (Marimastat, AG3340) and angiogenic inhibitors (angiostatin and endostatin) in patients with cancer have not produced obvious evidence of anti-metastatic, anti-invasive effects. These data indicate that other enzyme systems are needed to replace MMP at the invasion front of a tumor.

Cancer cell invasiveness *in vitro* can be a direct indication of a tumor's metastatic potential. Knowledge of the cell's invasive phenotype is important in developing cancer treatments that maximize patient survival and quality of life. It is also important in its use in formulating diagnostic tools for detecting cancer

progression and metastasis. Therefore, much effort has focused on measuring cancer cell invasiveness, a characteristic of the metastatic potential of carcinomas.

Invasiveness of a cell is often inferred by its cell surface proteolytic activities that degrade extracellular matrix (ECM) components, and that internalize ECM fragments. *In vitro* assays for such activities are often complicated by other cell surface phenomena such as adhesion, cell surface proteolysis, and membrane mobility. One particular assay designed to measure invasiveness of a cell involves the covalent linkage of fluorescence-labeled or radio labeled fibronectin (or other ECM components) to the surface of a cross-linked gelatin substrata (Chen et al., 1984, *J. Cell Biol* 98:1546-1555; Chen, et al., 1985, *Nature*, 316:156-158; Chen et al., 1989, *J. Exp. Zool.* 251:167-185; Chen et al., 1994, *J. Tiss. Cult. Meth.* 16:177-181; Meuller et al., 1989, *J. Cell. Biol.* 109:3455-3464). In this particular technique fibronectin was labeled and used to coat over-fixed protein film. The film was then used to measure cell surface proteolytic activities as well as the cellular invasive phenotype in terms of foci of invadopodial extensions and surface indentations in the film. However, this fibronectin-gelatin film assay is of limited value because (i) it uses conventional, over-fixed protein films; (ii) it lacks sensitivity for detecting proteolytic activity of moderately invasive cells such as most tumor cell lines in culture, fibroblasts and angiogenic endothelial cells, (iii) cross-linked gelatin fragments are not ingested by cancer cells; and (iv) it is difficult to build a three dimensional culture gel system from fibronectin and cross-linked gelatin materials. Thus, reliable procedures to measure the invasiveness of such cells will have significant impact in both clinical diagnostic and therapeutic applications of cancer.

The present invention provides a unique, functional based cell separation method to isolate various forms of cancer cells from blood, ascites and primary tumor tissue of patients with metastases, and peripheral blood mononuclear cells including endothelial cells from blood of normal donors. Additionally, the present invention provides evidence that seprase and/or DPPIV are selectively induced in invasive carcinoma cells and in activated fibroblasts (or other tissue cells) and sprouting endothelial cells of malignant tissues thereby providing targets for development of drugs for inhibiting tumor invasion and metastasis.

3. SUMMARY OF THE INVENTION

The present invention relates to a novel cancer cell capture system for rapid and efficient detection and selection of invasive cancer cells from the blood, ascites and/or tissue of cancer patients. In later stage cancer patients, some cancer cells were found associated with blood components to form large aggregates of cells. Such clumping of cells may contribute to organ dysfunction associated with late stage cancer. The present invention provides compositions and methods that can detect such cells in the blood of cancer patients with metastatic diseases, and may be used to remove such aggregates of cells as well as free-living individual cancer cells from the patient's blood, ascites and/or tissue.

In contrast to earlier antibody approaches for cancer cell isolation and detection, the present cell separation system is based on the functional properties of the cancer cells, *i.e.*, their ability to adhere to, degrade, and ingest the extracellular matrix. Thus, the cell separation and assay system of the prevent invention is designed to identify and isolate the very small fraction of cancer cells in the blood, ascites and tumor tissue of cancer patients that are viable, invasive and metastatic. The enriched cancer cell population can be used, for example, to determine their metastatic potential and the most effective treatment regime. The enriched cells may also be used in fusions with dendritic cells for cancer vaccine development.

The invention relates to natural fibrous compositions comprising blood-borne adhesion molecule-coated collagen, fibrin, cotton and plastic fibers, to be used as cell-adhesion matrices for use as blood filters by subjects having metastatic cancers. Type I collagen derived from, for example, placental tissues or rat-tail, is particularly useful for formation of the matrix which can be readily assembled into any form and be coated on vessel surfaces through cycles of polymerization and de-polymerization. The collagen matrix is coated with a variety of different adhesion molecules derived from blood, including but are not limited to fibronectin, laminin and vitronectin. Adhesion of individual cancer cells or cell clusters to such a cell-adhesion matrix provides a basis for cellular isolation.

The compositions can be used, for example, to remove undesired cancer cells and to enrich hematopoietic progenitor cells from the blood or bone

marrow for use as donor cells in bone marrow transplantation. Further, specific cancer cells of different carcinoma cancers may be enriched from whole blood by the cell separation methods of the present invention, subjected to *ex vivo* expansion, then used to interact with dendritic cells to develop an effective tumor vaccine. In addition, circulating cancer cells could be isolated from the patient and then subjected to a battery of chemotherapeutic regimes *in vitro*. Effective doses or drug combinations could then be administered to that same patient.

The compositions and methods of the invention provide a rapid method for detection and isolation of invasive cancer cells in blood, ascites and tissues of patients with cancer. Such cancers include, but are not limited to, prostate, breast, colon, lung, head & neck, brain, bladder, lymphoma, ovarian, renal & testis, melanoma, liver, pancreatic or other gastrointestinal cancers. Cancer cells are detected and characterized using the immunocytochemistry and cellular functional assays of the invention, i.e., collagen degradation and ingestion.

The method of the invention can be used to develop sensitive assays for the determination of invasive cancer cells in the blood, ascites or tumor tissue of cancer patients for use in prognostication, monitoring therapeutic and surgical treatments and early detection of cancer. The sensitivity and accuracy of measuring the metastatic potential of a cancer may be further enhanced using additional assays known to those of skill in the art, such as determining the tissue origin of cancer cells, measuring the angiogenic capability of the cells, and determining reduced leukocyte or complement association.

The cell-adhesion matrix of the invention also provides a cancer cell trap that allows for the high yield and efficient removal of viable cancer cells from whole blood, buffy coat, peripheral blood stem cell preparation or bone marrow. The cell separation method of the invention is intended for use in therapeutic apheresis and leukopheresis, in which autologous blood transfusions are done from which contaminating cancer cells have been removed. The present invention provides a highly efficient method for removal of cancer cells from whole blood of patients with prostate, breast, colon, lung, head & neck, brain, bladder, lymphoma, ovarian, renal & testis, melanoma, liver, or pancreatic and other gastrointestinal cancers.

Assays are also provided that may be used to screen for agents capable of inhibiting metastasis, thereby modulating the metastatic potential of cancer cells. Such assays involves contacting the cell-adhesion matrix with a cancer cell sample in the presence of a test agent and subsequent detection and quantitation of cancer cell adhesion to and degradation or ingestion of the matrix. The assay system of the invention can also be used to monitor the efficacy of potential anti-cancer agents during treatment. For example, the metastatic potential of cancer cells in whole blood can be determined before and during treatment. The efficacy of the agent can be followed by comparing the metastatic potential of the cancer cells throughout the treatment. Agents exhibiting efficacy are those which are capable of decreasing cancer invasiveness, increasing host immunity, and suppressing cancer proliferation, but having little or no effect on normal tissue cells. For example, such an anti-metastatic drug screening assay system has identified monoclonal antibodies and peptide inhibitors directed against seprase-DPPIV complex that block the ability of migratory cells to adhere, degrade, and invade a collagenous matrix (Figures 13 and 14). Furthermore, such assay system demonstrates that anti-sense and ribozyme constructs against seprase and DPPIV are capable of decreasing the invasiveness of breast carcinoma cells (Figure 19).

Methods and compositions are provided for inhibiting the metastatic potential of cancer cells comprising the administration of modulators of serine integral membrane proteases. This aspect of the invention is based on the observation that formation of a novel protease complex, comprising seprase and DPPIV, is a prerequisite for cell invasion into the collagen matrix. Such protease inhibitors include but are not limited to those capable of inhibiting the activity of DPPIV and seprase. The discovery that activation of seprase and DPPIV activity is a prerequisite for cell invasion and migration provides target molecules for drug screening assays designed to identify inhibitors of cancer cell migration.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-B. Composition and preparation of a cell-adhesion matrix.

Figure 1A. Preparation of the type I collagen based, cell-adhesion matrix, illustrating seven steps involved in matrix preparation, cell separation, and microscopic

measurement of the invasiveness of a cancer cell. Figure 1B. Utilization of the type I collagen based, cell-adhesion matrix in microtiter measurement of overall collagen degradation by the cells. The degradation and ingestion of the Bodipy-rhodamine collagen matrix by cancer cells are measured with a fluorescent microtiter plate reader as an increase in red fluorescence. The bottom graph shows release fluorescence unit (RFU) is proportional to the amount of Bodipy-rhodamine collagen overlaid on each microtiter well in the presence of bacterial collagenase at 37° C for 30 minutes.

Figure 2A-B. Diagnostic and therapeutic applications of the cell-adhesion matrix based, cell separation and assay system. Figure 2A. Cross-sectional views of cell separation and assay methods that may be used for diagnostic applications, including diagnostics, monitoring therapeutic or surgical responses, and early cancer detection. The steps of isolating, detecting and characterizing cancer cells from blood, ascites or tissue cell populations is illustrated. Figure 2B. Cross-sectional views of cell separation and assay methods of the present invention that may be used for therapeutic and preventive applications. The steps of isolating and culturing cancer cells from blood, ascites or tissue cell populations are illustrated.

Figure 3A-F. Cancer cell separation analyses of blood samples from patients and controls. Representative examples of fluorescent-tagged Hs578T breast carcinoma cells spiked in complete medium alone, or the blood of control normal donor and a bladder cancer (BLC) patient are shown. Red fluorescent-tagged Hs578T breast carcinoma cells were added to 3-ml of medium or blood samples, the cells were captured by a collagenous matrix in a microtiter well and cultured for 18 hours. The images shown were taken with super-imposed illumination of fluorescence and phase contrast, and represent only a portion of the microtiter well. Figure 3A. Fluorescent analysis of Hs578T breast carcinoma cells (500 cells) added to the medium alone. Estimated 495 cells were found as bright spots attached and spread on the matrix. Figure 3B. Fluorescent analysis of Hs578T breast carcinoma cells (500 cells) spiked in the blood of a normal donor. Estimated 415 red fluorescent carcinoma cells were found among the other blood cells (without fluorescent signal) in the culture that remained on the matrix substratum. Figure 3C. Fluorescent analysis of Hs578T breast carcinoma cells (19 cells) spiked in the blood of a normal donor. Estimated 11 red

fluorescent carcinoma cells were found among the other blood cells (without fluorescent signal) in the culture that remained on the matrix substratum. Figure 3D. Fluorescent image of Hs578T breast carcinoma cells (200 cells) spiked in the blood of a BLC patient. Estimated 182 Hs578T breast carcinoma cells along with circulating BLC cells were captured from blood of cancer patient. Hs578T cells tend to form aggregates with mononuclear blood cells of the patient seen as dark spots in this figure. Figure 3E. Fluorescent analysis of matrix-captured Hs578T breast carcinoma cells (19 cells) spiked in the blood of a BLC patient. Estimated 16 red fluorescent carcinoma cells were found among the other blood cells and one Hs578T breast carcinoma cell is seen in the field. Figure 3F. Immunofluorescent analysis of matrix-captured Hs578T breast carcinoma cell shown in Figure 3E and circulating BLC cells. Here, the Hs578T breast carcinoma cell and four bladder carcinoma cells derived from the blood were stained with fluorescein-conjugated anti-cytokeratins PCK antibody. The Hs578T breast carcinoma cell (shown as red fluorescence in Figure 4E) can be seen among the green fluorescent cytokeratin-labeled bladder carcinoma cells derived from patient's blood. Picture size A-D, 662 μm x 478 μm; Picture size E-F, 331 μm x 239 μm.

Figure 4A-I. Circulating cells attached to cell-adhesion matrix. Figure 4A-C. Circulating cells aggregated on a piece of tissue fragment in blood of a patient with squamous cell carcinoma of head and neck (HN). The cell-tissue cluster was captured on the substratum using a collagenous matrix, and cultured for four days (d4 in A), six days (d6 in B), and 13 days (d13 in C). Figure 4D. Cells aggregated on a piece of tissue fragment in blood of a patient with metastatic prostate cancer (PC), and cultured for three days (d3). Figure 4E. Circulating carcinoma cells from a patient with metastatic prostate cancer (PC) aggregated on a fibrin fiber (fibrin), and cultured for three days (d3). Figure 4F. Circulating cells from a patient with metastatic prostate cancer (PC) aggregated on plastic scraps (plastic), and cultured for three days (d3). Putative cancer cells of large size can be seen to preferentially adhere on plastic scraps in the field. Figure 4G. Circulating cells in the same culture as in F, but cultured for twelve days (d12). Putative cancer cells of large size can be seen to preferentially adhere on plastic scraps in the field. Figure 4H. Circulating cells from a

patient with metastatic prostate cancer (PC) aggregated on purified cotton (cotton), and cultured for three days (d3). Figure 4I. Growth of putative cancer cells from a patient with brain cancer (BN) aggregated on purified cotton (cotton), and cultured for 20 days (d20). Picture size A-G, 662 μm x 478 μm; Picture size H-I, 1324 μm x 956 μm.

Figure 5A-H. Circulating cells attached to type I collagen-based, cell-adhesion matrix. Figure 5A-C. Cell aggregates from a patient with metastatic pancreatic cancer (PnC) captured on the substratum using the type I collagen matrix, washed, and cultured for five days (A), nine days (B), and 16 days (C) on the matrix. These illustrate phase-microscopic appearance of a type I collagen based, cell-adhesion matrix and carcinoma cells, that invade the matrix and grow on the plastic surface. Carcinoma cells, not present in blood of most normal donors, have distinct morphology and increase their cellular size and number in culture, while co-isolated leukocytes are small and reduce their number in culture. Figure 5D. Circulating cell aggregates from a patient with metastatic pancreatic cancer (PnC) captured on the substratum using the type I collagen matrix. Cells were eluted from the matrix and cultured in complete medium for seven days on plastic surface (plastic). Spindle-shaped cells associate with epithelial-shaped, carcinoma cells at the periphery of a cellular colony. Figure 5E-F. Invasion of stromal fibroblasts into a type I collagen substratum. Cells were dissociated and isolated from a tumor biopsy of a patient with squamous cell carcinoma of head and neck (HN). Two morphologically distinct, cell types were isolated on the surface of the matrix: HN squamous cells remain quiescent on the matrix surface (E), while stromal fibroblasts invade into the collagen gel (F), approximately 80 μm below the level seen in Figure 5E as measured by the fine stage adjustment dial of Nikon Eclipse TE300 inverted microscope. Figure 5G. Morphology of a type I collagen based, cell-adhesion matrix illustrating transmission electron-microscopic appearance of the matrix and its adherent cancer cells, that were isolated from the blood of a patient with metastatic colon cancer (CC). Figure 5H. A high magnification view of the collagen fibers shown in Figure 5H, illustrating transmission electron-microscopic appearance of the assembled collagen fibers. Picture size A-F, 662 μm x 478 μm; Picture size G-H, indicated in the bar area.

Figure 6A-D. Morphological basis of the invasive phenotype of circulating carcinoma cells. Figure 6A-B. Circulating cell aggregates from a patient with metastatic colon cancer (CC). Cells were freshly isolated and cultured for one day (d1) and nine days (d9). These illustrate phase-microscopic appearance of degraded holes in the type I collagen based, cell-adhesion matrix generated by carcinoma cells and characteristics of carcinoma cells, that have distinct morphologies, i.e., small in size on d1 (A) but large and epithelial shape on d9 (B). Carcinoma cells increase their cellular size and number in culture, while co-isolated leukocytes are small and reduce their number. Also, spindle-shaped cells associate with epithelial-shaped, carcinoma cells in colonies (B). Figure 6C-D. Circulating cell aggregates from a patient with metastatic stomach cancer (SC). Cells were captured on the substratum using the type I collagen matrix, and cultured for 19 days. Epithelial-shaped, carcinoma cells grow on plastic surface in degraded holes of collagenous film (C) or line the edge of collagen film (D). Picture size A-B, 1324 μm x 956 μm; Picture size C-D, 662 μm x 478 μm.

Figure 7A-D. Cells from control blood samples that are isolated by the cell separation method. Figure 7A. Cells isolated from the blood of a 39 year-old normal donor and their underlying type I collagen matrix. Very few cells survived after 7 days in culture, while the matrix membrane stayed intact. Figure 7B. Cells isolated from the blood of a patient with benign colon tumor (CTN) and their underlying type I collagen matrix. Very few cells were isolated, and the matrix membrane stayed intact. Figure 7C-D. Circulating cells of a patient with metastatic breast cancer (BC) and their underlying type I collagen matrix. Blood sample was collected during treatment with chemotherapeutic compounds. Relatively few cells were isolated and survived in d1 (C) and d4 (D) cultures, and the matrix membrane stayed intact. Picture size A-D, 662 μm x 478 μm.

Figure 8A-L. The cell separation and assay system for the cell invasive phenotype using fluorescently labeled collagen, and immunocytochemistry of circulating carcinoma cells using antibodies against epithelial markers. Cells were derived from the blood of patients with breast carcinoma (BC, A-C), squamous cell carcinoma of head-neck (HN, D-F), colon cancer (CC, G-I), prostate cancer (PC, K),

and a normal donor (Control, L), as well as the Hs578T breast carcinoma cell line (Hs578T, J). Cells were captured on the substratum using a rhodamine-collagen matrix, and cultured for one day (d1) or 63 days (d63). Figure 8A-C. Ingested rhodamine-collagen blotch as a marker for the invasive phenotype of circulating carcinoma cells in a patient with breast cancer (BC). Cells were seeded on the matrix for 18 hours and fixed for examination. Among numerous leukocytes in the background, individual and cluster of putative cancer cells (PH, phase contrast image shown in A) were shown to ingest rhodamine-collagen fragments, marked Col+ (red fluorescence shown in B). Super-imposed image shown in panel C demonstrates the exact match of rhodamine-collagen spots to putative cancer cells. These represent the enumeration of 2,067 Col^+ cells per ml of patient's blood. Figure 8D-F. Ingested rhodamine-collagen blotch as a marker for the invasive phenotype of circulating carcinoma cells in a patient with squamous cell carcinoma of head-neck (HN). A large cluster and individuals of putative cancer cells (PH, phase contrast image in D) were shown to be coincident with cells positively antibody-stained for broad-spectrum cytokeratin subunits (PCK^+, in E) and these loaded with rhodamine-collagen Col+ (red fluorescence in F). These represent the enumeration of 20,814 PCK^+ and 18,003 Col^+ cells, respectively, per c.c. of patient's blood. The high number of PCK^+ compared to these of Col^+ cells may be due to the presence of less viable cancer cells that are positive for antibody staining, and that adhere to the cell-adhesion matrix. Figure 8G-I. Ingested rhodamine-collagen blotch as a marker for the invasive phenotype of circulating carcinoma cells in a patient with colon cancer (CC). Clusters and individuals of putative cancer cells (differential interference contrast image in G) were labeled with antibodies against epithelial surface antigen (ESA^+; stained red in G). ESA^+ cells were shown to be coincident with these stained with antibodies against the endothelial marker factor VIII ($F8^+$; green fluorescent cells in H) and these ingested rhodamine-collagen fragment Col+ (red fluorescent cells in I). These represent the enumeration of 2,284 ESA^+, 7,308 $F8^+$, and 1,978 Col^+ cells, respectively, per ml of patient's blood. The high number of $F8^+$ compared to these of Col^+ or ESA^+ cells may be due to the presence of normal endothelial cells in the blood. Figure 8J. The Hs578T breast carcinoma cell line stained with anti-PCK

antibodies, as carcinoma (epithelial) marker control. Figure 8K. PC cells in culture for 63 days (d63) stained ESA^+. Note that these large cells contain multiple nuclei and increase their diameter over 5-folds as compared to d1 cells in G. Figure 8L. Rhodamine-collagen matrix that remains intact after culturing blood cells from a normal donor for one day (d1). Picture size A-C, 1324 μm x 956 μm; Picture size D-F, 331 μm x 239 μm; Picture size G-L, 662 μm x 478 μm.

Figure 9A-L. Angiogenic propensity of circulating carcinoma cells. Cells were derived from the blood of patients with squamous cell carcinoma of head-neck (HN, A-C; I-J), colon cancer (CC, D-F) and prostate cancer (PC, G-H). Cells were captured on the substratum using the collagen matrix, and cultured for 1-11 days as indicated by d1 or d11. Figure 9A-C. A large cluster and seven individual HN cells (phase contrast image shown in A) that were labeled strongly for $F8^+$ (B) and that ingested collagen fragment Col+ (C). The HN cells shown in phase contrast (PH) image (A) are closely associated with these in the same field that exhibited positive reactivity with F8 antibodies (B) and these ingested rhodamine-collagen matrix (C). These represent the enumeration of 42,495 $F8^+$ and 15,611 Col^+ cells, respectively, per c.c. of patient's blood. The high number of $F8^+$ as compared to Col^+ cells is due to the presence of $F8^+$ endothelial cells in the blood. Figure 9D-F. A subset of $CD31^+$ and $F8^+$ cells representing the Col+ CC cells. The cells on the rhodamine-collagen matrix were labeled with antibodies against the endothelial cell marker CD31 and fluorescein conjugated antibodies against the endothelial marker factor VIII (F8). The association of bright red stained $CD31^+$ cells with CC cells was shown in differential interference contrast (DIC) image (D). CC cells in the same field exhibited positive reactivity with F8 antibodies (E) and these ingested rhodamine-collagen matrix (F). These represent the enumeration of 11,693 $CD31^+$, 6,577 $F8^+$ and 2,558 Col^+ cells, respectively, per c.c. of patient's blood. The high number of $CD31^+$ and $F8^+$ as compared to Col^+ cells is due to the presence of $CD31^+$ and $F8^+$ endothelial cells in the blood. Figure 9G-H. PC cells incorporating acetylated LDL. The bright red stained epithelial surface antigen (ESA) positive cells shown in differential interference contrast (DIC) image (G) display the ingestion of the fluorescein-LDL (H). These represent the enumeration of 9,744 ESA^+ and 34,105 LDL^+ cells, respectively, per c.c.

of patient's blood. The high number of LDL^+ as compared to ESA^+ cells is due to the presence of other endothelial cells that incorporate acetylated LDL. Figure 9I-J. Primary cultured HN cells incorporating acetylated LDL. Note that only one specific cell toward the center incorporate both fluorescein-LDL and rhodamine-collagen as shown in the triple fluorescein-Rhodymenia-Hoechst image (J), suggesting that this cell represents a cell retaining the invasive phenotype after in culture for 12 days. Figure 9K-L. Capillary network development by circulating CC cells on collagen gel. Network formation (K) and cord-like structures (L) were observed 2 days after plating circulating CC cells on type I collagen gel, 0.5-mm in thickness. Picture size A-C; G-J, 331 μm x 239 μm; Picture size D-F; K-L, 662 μm x 478 μm.

Figure 10A-L. Immune cancer killing and growth of circulating cancer cells in culture. Cells were isolated from the blood of patients with colon (CC, A-E), prostate (PC, F-I), or bladder cancer (BLC, J-L) using the collagen matrix, and cultured in the medium containing 10-20% human plasma. Figure 10A-C. $CD34^+$ peripheral blood stem cells clustering with the $F8^+/Col^+$ CC cells. The association of bright red stained $CD34^+$ cells with CC cells was shown in the differential interference contrast (DIC) image (A). CC cells in the same field exhibited positive reactivity with F8 antibodies (B) and these ingested rhodamine-collagen (C). These represent the enumeration of 111,082 $CD34^+$, 7,673 $F8^+$ and 2,558 Col^+ cells, respectively, per c.c. of patient's blood. The high number of $CD34^+$ and $F8^+$ as compared to Col^+ cells is due to the presence of $CD34^+$ stem cells and $F8^+$ endothelial cells in the blood. Figure 10D-E. $CD45^+$ leukocytes clustering with the Col+ CC cells. The isolated cells on the rhodamine-collagen matrix were labeled with antibodies against the leukocyte common antigens CD45. The association of bright red stained $CD45^+$ leukocytes with CC cells was shown in the DIC image (D). CC cells in the same field exhibited the ingestion of the rhodamine-collagenous matrix (E). These represent the enumeration of 125,670 $CD45^+$ leukocytes and 1,827 Col^+ cells, respectively, per c.c. of patient's blood. Figure 10F-I. Sequential views of cytolysis of immune and cancer cell clusters from a patient with prostate cancer (PC) who exhibited favorable prognosis. Most cancer cells were attacked by leukocytes and became fragmented after one day in culture (F-H). However, leukocytes

disappeared after seven days in such culture, leaving behind a few PC colonies (I). Figure 10J-L. Cytolysis of bladder cancer (BLC) cells due to autoimmune plasma derived from the same patient. BLC cells were cultured in the presence of 10% autologous plasma, derived from the blood of the same cancer patient (au.plasma in J-K), or those from a normal donor (n. plasma in L). BLC cells became lysed and brown in color in the medium containing autologous plasma in the second day (au.plasma in J-K), while they stayed viable for over 6 weeks in the medium containing normal plasma (n.plasma in L), suggesting a role of auto-cancer antibodies in complement cytolysis and signifying the host immunity against metastasis. Picture size A-L, 662 μm x 478 μm.

Figure 11A-B. Gel Filtration Column Chromatography and Immunoblotting for Sepharase and DPPIV. Figure 11A. WGA-purified, detergent-soluble proteins derived from WI38 cells were separated by a gel filtration column of Sepharase 12 (Pharmacal-LKB, Piscataway, N.J.). Protein standards used to calibrate the column were vitamin B-12 (1.35-kDa), myoglobin (17-kDa), ovalbumin (44-kDa), gamma globulin (158-kDa), catalase (232-kDa), ferritin (440-kDa), and thyroglobulin (670-kDa). Figure 11B. Fractions were analyzed by immuno-dot blotting using mAbs against seprase and DPPIV. Seprase and DPPIV were found in 200-kDa (Fraction 17), 440-kDa (Fraction 14), and 670-kDa (Fraction 13) ranges, suggesting the presence of the seprase-DPPIV complex at 440-670 kDa sizes.

Figure 12A-D. Characterization of the Seprase-DPPIV Complex Derived from WI38 Human Embryonic Lung Fibroblasts. Figure 12A. Immunoprecipitation (Ip) of surface-biotinylated WI38 fibroblasts. Both mAbs; against seprase (D28) and DPPIV (E19) identified a RIPA-solubilized protein complex that contains two major bands at 170- and 200-kDa, indicative of seprase and DPPIV, respectively. Figure 12B. The seprase-DPPIV complex demonstrated by Ip and immunoblotting (Ib). Seprase- or DPPIV-immunoprecipitates isolated from WI38 RIPA lysates were confirmed in seprase- or DPPIV-immunoblots but not in β1 or β3 integrin blots, suggesting that the protease complex is not associated with β1 and β3 integrins in RIPA. Figure 12C. Gelatinolytic activity of the seprase-DPPIV complex. The protease complexes were analyzed by gelatin zymography in the

absence of Ca^{++} and in the presence of 2 mM EDTA. Both seprase and DPPIV immuno-isolates (Ip) exhibit a 170-kDa gelatinase (seprase) activity. Figure 12D. DPPIV proline-specific peptidase activity of the complex. Both seprase and DPPIV immuno-isolates (Ip) exhibit same peptidase activity using fluorescent Ala-Pro-AFC (7-Amino-4-Trifluoromethyl Coumarin) substrate overlay assay. No activity could be observed for αv, α2, α6 or β3 integrin or control immuno-isolates.

Figure 13A-E. Cell Migration in Collagen Gel and Collagen Degradation by Wound-Activated WI38 Fibroblasts. Figure13A. Morphology of WI38 at 1 h (a, b, c) and 18 h (d, e, f) after wounding of the cell monolayer (photographed while cells were alive). Panel a & d: phase contrast of WI38 at the interface between the wound edge and cell-free glass surface, showing that spindle-shaped cells migrated on collagen fibers at 18 h (d). Panel b & e: fluorescent collagen gels in same fields shown in panels a & d. Uniform layer of TRITC-labeled collagen is seen at 1 h (b) but local removal of fluorescent collagen occurred at the wound edge by activated migrating cells at 18 h (e). Panels c & f: microscopic superimposed image of left and middle panels. Bar=10μm. Figure 13B. Dose-dependent inhibition of cell migration by inhibitory mAb E19 (against DPPIV) but not by control mAb C37 (against a cell surface glycoprotein gp-90). Three experiments of 24 h monolayer wound models were used for each antibody. Cell migration was quantified by measuring the areas of cell advancement from the original wound edge. The values are mean ±SD. Figure 13C. Reversal of antibody inhibition of cell migration. All antibodies, mAb E19 (against DPPIV) and mAb C37 (against gp-90), were applied at 5μg per ml. After day 3, antibodies were removed and the antibody inhibitory effect was reversed. Figure 13D. Histograms of the local removal of fluorescent collagen gel by wound-activated cells. Collagen degradation was measured as areas of fluorescent collagen removal by migratory cells. mAb E19 (against DPPIV) inhibited collagen removal by migratory cells while control mAb C37 (anti-gp-90) did not. All antibodies were applied at 5 g p er ml. Three experiments were used for each antibody. The values are mean ± SD. Figure 13E. Collagen degradation by migratory cells in a sparse culture. Collagen degradation was measured by the release of fluorescent collagen peptides from a collagen gel by

migratory WI38 cells. Bacterial collagenase was used as a positive control for fluorescent peptide release. All antibodies were applied at 5μg per ml. Three experiments were used for each antibody. The values are mean ± SD.

Figure 14A-B. Attachment and Spreading of WI38 Cells on Collagen Substratum are Mediated Primarily by β1 Integrins but Not by DPPIV. Figure 14A. Inhibition of WI38 cell spreading on collagen substratum by mAb C27 (against β1 integrins) but not by mAb E19 (against DPPIV) or mAb C37 (anti-gp-90). Figure 14B. Inhibition of WI38 cell attachment to collagen substratum by mAb C27 (against β1 integrins) but not by mAb E19 (against DPPIV) or mAb C37 (anti-gp-90). Each value represents the mean of three separate determinations ± S.D. Duplicate experiments gave similar results.

Figure 15. Co-localization of Seprase and DPPIV at Invadopodia of WI38 Cells Migrating in Collagen Gels. Phase contrast image of invadopodia (indicated by open arrow) in a WI38 cell migrating in type I collagen gel (a). Immunofluorescent image of DPPIV in the invadopodia (indicated by open arrow) that was labeled directly with TRITC-mAb E19 against DPPIV(b). Immuno-fluorescence image of seprase in the invadopodia (indicated by open arrow) that was labeled directly with FITC-mAb D28 against seprase (c). Superimposed image of panels b and c, showing that seprase and DPPIV co-localized at the invadopodia (indicated by open arrow) of a WI38 fibroblast migrating in collagen gel (d). Bar=10μm.

Figure 16. Seprase and DPPIV Distribution in Connective Tissue Cells of Human Malignant Breast Carcinoma as demonstrated by immunohisto chemistry of serial sections of paraffin-embedded tumor tissue. Both seprase and DPPIV are present in fibroblast-like cells of connective tissue immediately adjacent to invasive breast carcinoma but not in that of distant normal tissues (not shown). Arrows indicate positive brown, seprase or DPPIV stains for fibroblasts. Clusters of carcinoma cells are also positive for seprase (large cell aggregate in middle and bottom panels) and for DPPIV (large cell aggregate in top panel). Paraffin sections of breast carcinoma and adjacent normal tissue were placed in the same slide and stained

with mAb D8 against seprase (middle and bottom panels) or mAb E26 against DPPIV (top panel). Bar=100 μm.

Figure 17. Distribution of Seprase and DPPIV in Mucosa Cells of Healing Human Gingival Wounds. Frozen sections of healing mucosa wounds at 3-day-old (a, b, c, d and g) and at 7-day-old (e, f and h) were stained with hematoxylin and eosin (a and e), with TRITC-mAb D28 against seprase (b-d, and f) or with mAb E19 against DPPIV followed by TRITC anti-rat secondary antibody (g and h). In 3-day-old wound (a-d, and g), connective tissue (CT) contained cells that were strongly stained with seprase (b, c, and d) and DPPIV (g) antibodies. A confocal microscope image shows seprase localization at invadopodia in the form of filopodia (arrows) and cell bodies of fibroblast-like cells in connective tissue (panel d). In 7-day-old wounds (e, f and h), seprase staining was found (f) in the wound granulation tissue (GT), while DPPIV did not (h). Dotted lines indicate the border between granulation and connective tissues. Letter E indicates wound epithelium; CT, connective tissue; FC, fibrin clot; and GT, granulation tissue. Bar=200 μm.

Figure 18. Co-localization of seprase and DPPIV in microvessel endothelial cells, fibroblasts, and carcinoma cells at the invasion front of human malignant breast ductal carcinoma. Formaldehyde-fixed, paraffin embedded malignant human breast ductal carcinoma samples were made in serial sections, seprase distribution was shown by brown staining using mAb D8 (left three panels) and DPPIV distribution on an adjacent section using mAb E26 (right three panels). Top two panels show normal breast tissues approximately 2 cm distance from tumor sites. Negative seprase and DPPIV stains were found in epithelial cells (black open arrows), fibrocytes (black arrows) and endothelial cells (black open arrowheads). Middle two panels indicate infiltrating sheets of poorly differentiated (high-grade) carcinoma cells with predominant brown cellular stains of seprase and DPPIV in tumor cells (orange open arrows), in fibroblasts (orange arrows), and in some endothelial cells (orange open arrowheads) but not in some larger vessel lining cells (black open arrows). Arrows in top and middle panels indicate a scale of 100 μm. Bottom two panels show low-magnification view of infiltrating sheets of poorly differentiated (high-grade) tumor cells with predominant brown cellular stains of

seprase and DPPIV in tumor cells (orange open arrows), in fibroblasts (orange arrows), and in some endothelial cells (orange open arrowheads). Seprase and DPPIV are specifically expressed in tumor cells at the invasion front as indicated by most tumor cells in the field but are absent in these in the center of tumors as indicated in the center of the field. Arrows in bottom panels indicate a scale of 800 μm.

Figure 19. Relationship of protease expression and collagen-degrading ability of MDA-MB-436 cells. **A**, Release of fluorescent collagen peptides by MDA-MB-436 cells differentially expressing seprase and DPPIV. Collagen degradation was measured by the release of fluorescent collagen peptides by parental cells (Parent), cells transfected with vector (pA11), cells transfected with seprase cDNA (pA15) and cells transfected with DPPIV ribozyme (pZ8). Three experiments were used for each and the values were mean ± SD. Star "*" indicates ($\rho < 0.05$ for pA15 seprase sense and pZ8 DPPIV ribozyme transfectants as compared to parent and pA11 vector transfected cells. **B**, Differential expression of seprase and DPPIV in above-mentioned MDA-MB-436 cells. Cells were extracted with RIPA buffer and immunoblots were done using mAb D8 (Anti-seprase) and mAb F4 (Anti-DPPIV).

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a novel method for forming a cell-adhesion matrix for isolation and detection of a subpopulation of cancer cells which are viable, invasive, and metastatic from a sample derived from a subject possessing a cancer. In particular, a method is described that utilizes a collagenous matrix system for isolation and detection of cancer cells. The invention described in the subsections below further relates to a novel highly sensitive *in vitro* assay for measuring the metastatic potential of a cancer. An important feature of the assay includes applicability to cancer cells in blood, bone marrow, ascites, body fluid, and tumor tissue, or any established cancer cell line.

The "cell-adhesion matrix" of the invention provides a cancer cell trap that allows for the high yield and efficient removal of viable cancer cells from whole blood, buffy coat, peripheral blood stem cell preparation or bone marrow. The cell separation method of the present invention may be used for cancer diagnostic purposes, e.g. early detection, monitoring therapeutic and surgical responses, and

prognostication of cancer progression. The cell separation method of the present invention may also be used for cancer preventive and therapeutic purposes, including the use in blood filter as a cancer cell trap, in genetic and cytogenetic analyses, in new drug target discovery, and in cancer vaccine development.

The *in vitro* assay system of the invention further provides a screening method for identifying agents with anti-metastatic activity. Such agents may be used to inhibit metastatic spread of cancer cells in subjects having cancer. In addition, the assay may be used to screen for nucleic acid molecules with anti-metastatic potential. For example, antisense and ribozyme molecules may be screened for their ability to inhibit metastasis. In yet another embodiment of the invention, the activity of seprase or DPPIV may be measured to determine the metastatic potential of cancer cells.

5.1 THE MATRICES OF THE INVENTION

The present invention provides a novel, natural, cell-adhesion matrix system that may be used for the isolation and detection of cancer cells from samples derived from cancer subjects. The natural cell-adhesion matrix of the invention has specific binding affinity for blood-borne cell adhesion components, including but not limited to fibronectin, laminin and vitronectin. The present invention is based on the observation that cancer cells present in the circulation of patients with metastatic diseases can attach to tissue fragments and form large cellular clusters indicating that natural structural scaffolds have a high affinity for blood-borne adhesion molecules and, therefore, promote attachment of metastasized cancer cells. Additionally, it was discovered that natural fibers, including type I/III collagen, fibrin, purified cotton, and mechanically scratched surfaces of tissue culture plastic, absorb preferentially blood-borne adhesion components that promote adhesion of cancer cells.

The present invention encompasses cell-adhesion matrices comprising collagenous fibers, fibrin gels, purified cotton or plastic fibers for use as a cellular substratum for the detection and isolation of viable human cells *in vivo*, these cells include but not limited to carcinoma, endothelial and tissue cells. A variety of commercially available collagenous materials may be used to prepare the collagen matrix, including, but not limited to, human placental type I collagen, purchased from Calbiochem-Novabiochem Co. (La Jolla, CA); and rat-tail type I collagen, available

from Collaborative Biomedical Products (Becton and Dickinson Labware, Bedford, MA). Any form of recombinant collagen may also be employed, as may be obtained from a collagen expressing recombinant host cell, including bacterial, yeast or mammalian cells.

Type I collagen can be readily assembled and reassembled into any form and be coated on vessel surfaces through cycles of polymerization and de-polymerization. For example, it is well-known that type I collagen monomers, at a concentration of over 0.3 mg/ml, polymerize under conditions of natural pH, warm temperatures (25 – 37° C) and medium containing saline, and that collagen fibers de-polymerize in the condition of pH 2-4, cold temperatures (2-10° C), and low salt medium (Klasson, S.C., et al. 1986, Coll. Relat. Res. 6:397). Such a cell-adhesion matrix can be formed lining the wall of a micro capillary, cell isolation columns, tissue culture plates, or micromesh for capture of cancer cells in biological fluids.

The matrix scaffold of the present invention may be composed of natural fibers, including, but not limited to, collagens, fibrin and purified cotton. A common property of these matrix scaffolds is that their surfaces have affinity for blood-borne adhesion molecules, including, but not limited to, fibronectin, vitronectin and laminin. The matrix scaffold, when coated with cell adhesion molecules present in whole blood, plasma or serum, provides an adhesive surface which supports the attachment of cancer cells and tissue cells from high-density cell populations. These populations may be derived from blood, lymph, bone marrow and tumor tissue and may include many different cell types.

The method of preparing a collagenous matrix is described in detail in this application. A matrix scaffold composed of fibrin fibers were prepared on vessel's surfaces by reducing the anti-coagulant content of plasma. Animal or human plasmas were initially diluted to 10-20% with Dulbecco's modified Eagle's medium (DMEM) and added into cell isolation vessels or wells. The vessels or wells were incubated for 30 min at 37° C in a CO_2 incubator to allow polymerization of the fibrin fibers on vessel's surface. Purified cotton fibers were simply suspended in DMEM containing 10-20% bovine serum and seeded in cell culture wells for coating of cell adhesion molecules. Similarly, glass and plastic fibers were coated with cell adhesion

molecules by suspension in DMEM containing 10-20% bovine serum. A cell culture plate was scrapped with an Eppendorf peptide tip to prepare as a matrix scaffold. It should be noted that pre-coating of cell adhesion molecules to a matrix scaffold is not necessary when whole blood or a buffy coat will be applied to a matrix scaffold. However, whole blood or buffy coat should be treated with anticoagulants to prevent coagulation during the cell separation procedure. Specifically, blood or buffy coat were pre-diluted with equal volume of medium containing 0.5 mM EDTA or with 10% anticoagulant citrate dextrose (ACD; Baxter Healthcare Corporation, IL) containing 50 unit heparin/ml.

To facilitate detection of cancer cells bound to the collagenous matrix and to measure the metastatic potential of such cells, the matrix can be labeled with a variety of different agents, including, but not limited to, fluorescent dyes, biotin, color dyes , and radioactive probes. For example, collagen fibers can be labeling by direct conjugation of dyes thereby protecting polymerization sites in the collagen from labeling . Collagen fibers, either labeled by this method or unlabeled, can be readily disassembled into soluble collagen monomers, which can be subsequently assembled into any form on vessel surfaces. Preferred dyes for labeling of collagen include Bodipy-rhodamine or fluorescein dyes, available from Molecular Probes, Inc., which are quench-fluorescent dyes. As illustrated in Figure 1A and 1B, Bodipy-rhodamine's fluorescent signal (yellow) is reduced when two dye molecules are situated close together on collagen but is increased (red) when collagen is degraded or cleaved by enzymes to separate two dye molecules. Methods for labeling of the matrix are well known to those of skill in the art. Likewise, conjugation of a cytotoxic compound to such collagen fiber can provide a novel vehicle for delivery of the drug to specific cancer cells. For example, injection of a cytotoxin-conjugated, collagen microsphere into a patient with cancer can achieve specific killing of cancer cells.

The surface of a matrix scaffold is further coated with blood-borne adhesion molecules. A variety of commercially available solutions containing blood-borne adhesion molecules can be used to coat the matrix, including, but not limited to, calf serum, fetal calf serum (Collaborative Research, Inc., Bedford, MA); human serum (Sigma); and human plasma fibronectin, laminin and vitronectin

(Collaborative Research, Inc., Bedford, MA). The surface of the matrix scaffold can be coated with adhesion molecules using either 10-20% of calf serum, fetal calf serum, or human serum (or plasma), or 0.01 - 0.5 milligram per milliliter of human plasma fibronectin, laminin and vitronectin.

The present invention achieves a highly desirable objective, namely providing a method for prevention and intervention of metastases formation in a cancer subject by a cell separation system. Specifically, the invention encompasses a method for isolation of cancer cells derived from a cancer subject comprising:

(a) inoculating a cancer cell sample derived from a cancer subject onto cell-adhesion matrix;

(b) incubation of the cancer cell sample for a time sufficient to allow adhesion of cancer cells to the matrix, followed by removal of non-adherent cells; and

(c) *ex vivo* propagation of cancer cells bound to the matrix.

The cell separation method of the present invention may be used to isolate any desired cancer cell population from *in vivo* or *in vitro* sources including, but not limited to, body fluids, e.g. circulating blood, urine, bone marrow, spinal and pleural fluids, ascites, sputum; dissociated tumor tissue specimens; and cultured tumor cells. Examples of desired cancer cell populations include, but are not limited to, carcinoma cells of prostate, breast, colon, brain, lung, head & neck, ovarian, bladder, renal & testis, melanoma, lymphoma, liver, pancreatic and other gastrointestinal cancer. Specifically, desired cancer cell population from various cancers include lung carcinoma cells, lung adenoma cells, colon adenocarcinoma cells, renal carcinoma cells, rectum adenocarcinoma cells, ileocecal adenocarcinoma cells, gastric adenocarcinoma, pancreatic carcinoma, hepatoma cells, hepatocellular carcinoma cells, prostate adenocarcinoma cells, bladder carcinoma cells, breast carcinoma, ovarian teratocarcinoma, amalanotic melanoma cells, malignant melanoma cells, squamous cell carcinoma of the cervix, larynx and of oral origin; glioblastoma cells, endometrial adenocarcinoma, astrocytoma, Burkitt lymphoma cells, and non Hodgkin's lymphoma cells.

The presence of cancer cells bound to the matrix can be detected using a variety of different methods including the use of functional, immunophenotypic and cytomorphologic features of neoplastic cells. Additionally, any bound cell may be detected based on the ingestion of the labeled collagenous matrix by the cells.

5.2 THERAPEUTIC AND PREVENTIVE APPLICATIONS IN THE PRESENT INVENTION

The present invention provides cell separation methods that may be used, for example, to remove undesired cancer cells and to enrich hematopoietic progenitor cells from the blood or bone marrow for use as donor cells in bone marrow transplantation. Following cell separation methods, antibodies reactive with carcinoma epitopes such as epithelial markers, or antibodies reactive with endothelial cells, such as anti-CD31 or anti-CD34, can be used to enrich for cancer or endothelial cell populations, respectively.

In a specific embodiment, cancer cells derived from different cancers may be enriched from whole human blood by the cell separation methods of the present invention, subjected to *ex vivo* expansion, then used to interact with dendritic cells to develop an effective tumor vaccine using procedures including a method described (Brugger et al., 1999, Annals of the New York Academy of Science 872:363-371).

In another embodiment of the invention, circulating cancer cells can be isolated from a cancer subject and then subjected to a battery of chemotherapeutic regimes *in vitro* to determine the efficacy of a specific treatment. Effective doses or drug combinations could then be administrated to that same subject (designer drugs).

In another embodiment of the invention, a cytotoxin-conjugated collagen microsphere can be injected into a patient with metastatic cancer for selective killing of cancer cells. Specific adhesion and internalization of toxic collagen by cancer cells *in vivo* may provide a novel treatment.

The invention also provides fibrous compositions, including but not limited to blood-borne adhesion molecule-coated collagen, fibrin, cotton and plastic fibers, to be used as cell-adhesion matrices for use as blood filters by subjects having metastatic cancer. The use of such a cell-adhesion matrix involves the perfusion of

the subject's blood through the cell-adhesion matrix. In the blood perfusion protocol, the subject's blood is withdrawn and passes into contact with the cell-adhesion matrix. During such passage, cancer cells present in the patient's blood, preferentially adhere to the cell-adhesion matrix and are removed from the circulation of the patient.

To form the cell-adhesion matrix, collagen or the above-mentioned material is formed within a vessel, including but not limited to columns, tissue culture plates, microcapillaries, or micromesh for capture of cancer cells in biological fluids. The vessel contains an input and output outlet for passage of the subject's blood through the containment vessel. In a specific embodiment of the invention, the cell-adhesion matrix may be formed within a containment vessel which includes a blood input line, which is operatively coupled to a conventional peristaltic pump. A blood output line is also included. Input and output lines are connected to appropriate arterial venous fistulas, which are implanted into, for example, the forearm of a subject. Alternatively, apheresed peripheral blood can be applied in conjunction with the above-mentioned cancer cell isolation by the cell-adhesion matrix. Apheresis was initiated upon recovery of the white blood cell count to equal or more than 1.times.10.sup.9 /L. Apheresis or leukopheresis can be performed using a Cobe Spectra Cell Separator (Lakewood, Colo.) at a rate of 80 ml/min for 200 min (total volume of 16 L). The use of the cell-adhesion matrix of the invention provides a novel method that can remove cancer cells from the circulation of a patient in which cancer cells have great potential to metastasize.

5.3 ASSAYS FOR MEASURING METASTATIC POTENTIAL OF CANCER CELLS

It is an object of the present invention to provide a method for the identification of subjects possessing a cancer with an increased metastatic potential. The present invention relates to evaluation of metastatic potential by detecting the ability of a subject's cancer cells to invade, *i.e.*, adhere, degrade and ingest a collagenous matrix, using the collagen based cell-adhesion matrix system of the present invention. The detection and measurement of the cancer cell's angiogenic propensity, cell viability and proliferation constitute additional novel strategies for prognosis of cancer.

The cell separation and assay methods described herein may be used for a variety of diagnostic uses. These uses include morphological, molecular, biochemical or immunological assays in early detection, in monitoring therapeutic and surgical response, and in prognostication of cancer progression. For example, RNA may be prepared from circulating cancer cells and subjected to real-time polymerase chain reaction (PCR), DNA microarray analysis and serial analysis of gene expression (SAGE) and to identify differentially expressed genes that are diagnostic markers for cancer or drug targets for controlling metastasis. Furthermore, protein profile of isolated cancer cells can be done by proteomics analyses to discover novel metastatic markers and drug targets. The isolated cancer cells may be stained for epithelial specific antigens, seprase and DPPIV antigens or endothelial cell markers, and quantitated to determine the number of cancer cells present for evaluating the metastatic potential of a cancer. The isolated cancer cells may be assessed morphologically as a routine pathological evaluation of a cancer. The isolated cancer cells may be cultured under sterile conditions and subjected to cytogenetic analysis to detect the presence of chromosomal abnormalities and mutation determination. The isolated cancer cells may be reacted with molecular probes for more sensitive detection of mutation using DNA microarray, PCR and FISH. The methods thereby avoid the use of invasive and expensive surgical procedures heretofore relied upon for such a determination.

The present invention achieves a highly desirable objective, namely providing a method for the prognostic evaluation of subjects with cancer and the identification of subjects exhibiting a predisposition to developing metastatic cancer. Specifically, the invention encompasses functional assays for determining the metastatic potential of cancer cells isolated from a subject using the cell-adhesion matrices of the present invention.

Specifically, the invention encompasses a method for determining the metastatic potential of cancer cells derived from a cancer subject comprising:

(a) inoculating a cancer cell sample derived from a cancer subject onto a cell-adhesion matrix;

(b) incubation of the cancer cell sample for a time sufficient to allow adhesion to, migration across or ingestion of the matrix by the cancer cells to occur; and

(c) detection of adhesion to, migration across or ingestion of the material by the cancer cells wherein detection of cancer cell adhesion to, migrationacross or ingestion of the material is an indicator of cancer cells with metastatic potential.

The assay of the invention involves the creation of an artificially generated matrix onto which cancer cells are inoculated. Cells which may be inoculated onto the matrix include but are not limited to blood cells, cancer cell lines and cells derived from the tumor of a mammal, including cells derived from the tumor of a cancer subject, *i.e.*, biopsied tumor tissue, etc..

Once the cancer cells have been inoculated onto the matrix, the matrix is incubated for a time sufficient to allow adhesion, ingestion or invasion to occur. The inoculation time will vary depending on the metastatic potential of the inoculated cancer cells.

Following incubation, cancer cell adhesion to the matrix and/or ingestion of the matrix by cancer cells is detected using a variety of different methods. For example, a collagenous matrix may be labeled with agents such as, for example, flourescent dyes, biotin, color dyes and radioactive probes. Ingestion of the matrix by the cancer cells results in labeling of the cells. The detection of cancer cells expressing the labeled markers may be accomplished using a variety of different methods well known to those skilled in the art including but not limited to fluorescence microscopy, fluorescent activated cell sorting (FACS) or scintillation counter measurement of radioactivity. Using such labels, the level of cell adhesion and ingestion of matrix material may be quantitated to determine the metastatic potential of the cells.

In addition, the metastatic potential of cancer cells isolated using the matrices of the invention may be determined using a variety of different assays well known to those skilled in the art. For example, the cancer cell sample can be incubated with labeled acetylated low density lipoprotein (acLDL+), plated on a

collagen gel for capillary network formation, or stained with antibodies directed against endothelial cell markers, including but not limited to CD31+, Flk1+, and factor VIII (F8+) for a time sufficient to allow measurement of the acquirement of the endothelial phenotype by cancer cells to occur. Alternatively, the propensity of the cancer cells to undergo apoptosis may be measured using an apoptosis assay kit (Molecular Probes, Inc.). The cancer cell clusters may be reacted with antibodies directed against leukocyte markers, including but not limited to CD45, CD19, CD8, and CD4 for a time sufficient to allow numeration of cancer cell viability, association with immune cells (leukocytes, T-cells and killer cells) and resistance to cellular or complement cytolyses. The ability of isolated cancer cells to form colonies in culture can also be determined by propagating the cancer cells in tissue culture medium containing 10-20% human plasma for 1-5 weeks thereby allowing the cancer cells to form clones.

The assay system of the invention can also be used to monitor the efficacy of potential anti-cancer agents during treatment. For example, the metastatic potential of cancer cells can be determined before and during treatment. The efficacy of the agent can be followed by comparing the number or metastatic potential of the cancer cells throughout the treatment. Agents exhibiting efficacy are those, which are capable of decreasing the level of detectable cancer cell adhesion, degradation or ingestion of a collagenous matrix, the cancer cell viability, and the colony-forming ability.

5.4 IN VITRO SCREENING ASSAY FOR IDENTIFICATION OF ANTI-METASTATIC AGENTS

The present invention further provides screening assays for identification of agents capable of inhibiting the spread of cancer cells from a primary tumor to a site of metastasis formation. In accordance with the invention, agents may be screened for their ability to inhibit metastasis of cancer cells. In utilization of the assay of the invention for purposes of identifying anti-metastatic agents, the test agent is co-inoculated with cancer cells onto the collagenous matrix. The adhesion, ingestion and/or degradation of the collagenous matrix by cancer cells in the presence of a test agent is compared to the adhesion, ingestion and/or degradation of the

collagenous matrix in the presence of a vehicle control, wherein an anti-metastatic agent is identified as one capable of inhibiting the adhesion, ingestion and/or degradation of the collagenous matrix by cancer cells.

Specifically, the invention comprises a method for identifying an agent that inhibits metastasis of cancer cells comprising:

(a) inoculating a cancer cell sample and either a test agent or a vehicle control onto the cell-adhesion matrix

(b) incubation of the matrix for a time sufficient for adhesion, degradation and/or ingestion of the matrix by the cancer cells to occur; and

(c) detecting the adhesion and/or ingestion of the matrix by the cancer cells,

wherein a decrease in the adhesion, ingestion and/or degradation of the collagenous matrix by the cancer cells in the presence of the test agent, as compared to the number of cancer cells detected in the presence of a vehicle control, identifies a compound that inhibits metastases formation.

The agents which may be screened in accordance with the invention include, but are not limited to inorganic compounds, peptides, antibodies and fragments thereof, and other organic compounds (e.g., peptidomimetics) that are capable of inhibiting the spread of cancer cells from a primary tumor to a site of metastases formation. Agents may include, but are not limited to, peptides such as, for example, soluble peptides, including but not limited to members of random peptide libraries; (see, e.g., Lam, K.S. et al., 1991, Nature 354:82-84; Houghten, R. et al., 1991, Nature 354:84-86), and combinatorial chemistry-derived molecular library made of D- and/or L- configuration amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate directed phosphopeptide libraries; see, e.g., Songyang, Z. et. al., 1993, Cell 72:767-778).

Agents identified via assays such as those described herein may be useful, for example, in defining the properties of cancer cells that enable successful migration and invasion, and for inhibiting metastases formation in cancer subjects. Assays for testing the efficacy of compounds identified in the screens can be tested in

animal model systems for metastasis formation. Such animal models may be used as test substrates for the identification of drugs, pharmaceuticals, therapies and interventions which may be effective in treating cancer metastasis.

The assay of the invention may also be used to screen for nucleic acid sequences capable of inhibiting the metastatic potential of cancer cells. Such nucleic acid molecules include molecules comprising protein coding sequences or anti-sense sequences. The nucleic acid molecules may be transferred to cancer cells prior to assaying by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection.

In accordance with the invention, an assay system can be used to screen for agents that modulate the activity of serine integral membrane proteases, including DPPIV and seprase and thereby modulate the metastatic potential of cancer cells. To this end, cells that endogenously express DPPIV and seprase can be used to screen for agents. Alternatively, cell lines, such as 293 cells, COS cells, CHO cells, fibroblasts and the like are genetically engineered to express DDPIV and seprase for use in screening. Preferably, host cells genetically engineered to express DDPIV and seprase can be used as an endpoint in the assay; e.g., as measured by a chemical, physiological, biological or phenotypic change.

In utilizing such cell systems, the cells expressing the DDPIV and seprase are exposed to a test compound or to a vehicle control. After exposure, the cells can be assayed to measure the expression and/or activity of DDPIV and seprase protease activity. For example, after exposure, cell lysates can be assayed for gelatinolytic activity (seprase), or substrates containing phosphorylated residues adjacent to proline, such as H-Gly-Pro-*p*-nitroanilide or amino methylcoumarin (DPPIV and seprase) and Z-Gly-Pro-*p*-nitroanilide or amino methylcoumarin (seprase) (Kaspari et al., 1996). The ability of a test compound to decrease levels of DPPIV and/or seprase protease activity, below those levels seen with cells treated with a vehicle control, indicates that the test compound inhibits DDPIV and seprase associated protease activity.

In addition, assays may be used to identify agents that antagonize the interaction between DPPIV and seprase, thereby inhibiting the activity of the protein

complex. To identify such agents, DPPIV and seprase proteins are incubated in the presence and absence of a test compound followed by detection of complex formation. A decrease in complex formation indicates the identification of an agent capable of antagonizing the interaction between DPPIV and seprase.

In one aspect of the invention, soluble DPPIV and seprase may be recombinantly expressed, labeled and utilized in none-cell based assays to identify compounds that inhibit the interaction between DPPIV and seprase. In such assays, either the DPPIV or seprase can be attached to a solid substrate such as a test tube or microtitre well, by means well known to those known in the art. The test agents are then assayed for their ability to inhibit the interaction between DPPIV or seprase on the solid substrate.

5.5 COMPOSITIONS AND USES

The present invention relates to a novel cancer cell capture system for rapid and efficient detection and selection of invasive cancer cells from the blood and/or tissue of cancer patients. The invention relates to fibrous compositions comprising blood-borne adhesion molecule-coated collagen, fibrin, cotton, and plastic fibers, to be used as cell-adhesion matrices. The invention further relates to use of such coated fibers to detect the presence of metastatic cells in cancer patients, and to filter the blood of subjects having metastatic cancers. Cell-adhesion matrices that may be administered to subjects with cancer include those described in Sections 5.1.-5.2., supra.

The present invention also provides cellular compositions that can be isolated by the cell-adhesion matrix of this invention. Such cellular compositions comprise a small subpopulation of tumor cells that have potential to metastasize, as well as cancer cell clusters in the blood that may contribute to organ dysfunction associated with late stage cancer. The enriched cancer cell population can be used, for example, to determine novel drug targets, genetic defects involved, their metastatic potential and the most effective treatment regime. The enriched cells may also be used in fusions with dendritic cells for cancer vaccine development.

The present invention provides for treatment of proliferative disorders such as cancer, by administration of agents that regulate the metastatic activity of

cancer cells. Compounds that may be administered to subjects with cancer include those agents identified using the assays described in Sections 5.3., *supra*.

In a specific embodiment of the invention, key proteases required for the tissue invasive phenotype were identified. For example, activation of serine integral membrane proteases, such as seprase and DPPIV were shown to be required for tissue cell migration and invasion. Thus inhibitors of such proteases may be used to inhibit the metastatic activity of cancer cells. Such inhibitors include antibodies immunospecific for seprase or DPPIV (Figure 13A-E). Alternatively, transfection into cancer cells of a vector expressing antisense RNA or ribozymes for seprase or DPPIV may be used to reduce the ability of cancer cells to metastasize (Figure 19).

In addition, protease inhibitors may be administered to inhibit the spread of cancer cells from a primary tumor to a site of metastasis formation. As demonstrated in Section 7, Example 2, an inhibitor of DPPIV, *i.e.*, an anti-DPPIV antibody, was capable of inhibiting cancer cell migration. Cancers involving metastasis of tumor cells to other locations in the body are treated by administration of an agent that inhibits cancer cell migration and tissue invasion. Such cancers include, for example, carcinomas, such as breast cancer and prostate cancer.

Compounds identified for use in prevention of cancer cell metastasises can be tested in suitable animal model systems prior to testing in humans, including but not limited to dogs, rats, mice, chicken, cows, monkeys, rabbits, etc. For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used.

In specific embodiments, compounds that inhibit cancer metastasis are administered to a subject having cancer where it has been determined that the subject's cancer cells have an increased metastatic potential. The increased metastatic potential can be readily detected, *e.g.*, by obtaining blood sample (or biopsy tissue from a patient) and assaying for the metastatic potential of the isolated cancer cells using the cell separation and assay system of the present invention.

The invention provides methods of treatment of cancer by administration to a subject of an effective amount of a compound that inhibits the

metastatic potential of cells. In a preferred aspect, the subject is an animal, and is preferably a mammal, and most preferably human.

The present invention also provides pharmaceutical compositions that can be linked to the cell-adhesion matrix or its isolated cellular component of this invention. Such compositions comprise a therapeutically effective amount of the compound capable of regulating the migration and invasion of cancer cells, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions.

6. EXAMPLE: ISOLATION OF CIRCULATING CARCINOMA CELLS INVOLVED IN ANGIOGENESIS AND METASTASIS

The data provided below, demonstrates that viable carcinoma cells can be isolated from peripheral blood of cancer patients with metastatic diseases by novel cell-adhesion matrix cell separation and assay methods, and these cells acquire molecular determinants necessary for angiogenesis and metastasis to occur. Comparing to the cells derived from primary tumor, such viable circulating cancer cells represent a small subset of cancer cell population that have potential to metastasize.

6.1 MATERIALS AND METHODS

6.1.1 COLLECTION OF BLOOD SAMPLE

Blood or cells collected from an *in vivo* source are subjected to cell isolation within a relatively short time after their collection because the cells may lose their viability. In order to maintain the optimal isolation of cancer cells, it is preferred that blood, ascites or tissue samples are stored at 4° C and used within 24 hours after their collection, most preferably, within four hours. Approximately 10-20 ml of blood each time were collected in Vacutainer tubes (Becton Dickinson, green top, each tube holds 7-ml) containing Lithium Heparin as anticoagulant. Patient age, sex, date of diagnosis, therapeutic interventions, clinical status, and biopsy report were retrieved from the patients' charts. The protocol was approved by the institutional review boards.

6.1.2 ISOLATION OF CANCER CELLS FROM BLOOD OR TUMOR TISSUE

The collagen polymerization solution was prepared and adjusted to a pre-determined concentration, from 1 to 2 milligrams per milliliter for collagens in Dulbecco's modified Eagle's medium (DMEM), on ice immediately prior to the gelation into vessel substrata (Figure 1A). Specifically, type I collagen solution (rat-tail type I collagen, 4.0 mg/ml, Collaborative Biomedical Products, Becton and Dickinson Labware, Bedford, MA) was mixed with equal volume of DMEM at 4° C. The mixture was overlaid as a thin layer onto the bottom of 96-well microtiter or 6-well culture plates (Nunc, Inc., Naperville, IL) at 4° C. To form a gel of collagen fibers, plates were incubated for 30 minutes at 37° C to allow polymerization of collagen layer.

6.1.3 ISOLATION OF CANCER CELLS FROM BLOOD, ASCITES OR TUMOR TISSUE

To separate cancer cells from a cell population, the collagen matrix was first coated with the cell culture medium, consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and RPMI1640 supplemented with 10% calf serum, 15% Nu-serum (Collaborative Research, Inc., Bedford, MA), 2 mM

L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 1 unit/ml penicillin, and 10 μg/ml streptomycin. However, cancer cells can be isolated directly from whole blood without the need to coat additional adhesion molecules, which are present in the blood.

The samples were processed for plasma and cell isolation at 4° C. Blood samples were centrifuged to collect plasma, and cell pellets were diluted with PBS containing 2% bovine serum and 0.5 mM EDTA to the original volume and then fractionated using Ficoll-Paque (Pharmacia) to collect mononuclear cells. The mononuclear cell fraction was further selected for viable and invasive carcinoma cells by differential adhesion to the cell-adhesion matrix. Briefly, mononuclear cells were suspended in the complete culture medium (to same volume as the blood). A portion of mononuclear cells were seeded, i.e., 0.1-ml per well of 96-well microtiter plate or 1 to 10 ml per well of 6-well tissue culture plate (NUNC) that were coated with the collagen matrix, for 15 minutes to 1 hour. The culture was washed gently with medium to remove non-adherent cells. For immunocytochemistry and functional assays, cells in microtiter plates were cultured for 12-24 hours before processing. For cell separation studies, cells adherent on the matrix were then suspended with trypsin/EDTA solution (GIBCO) for 5 minutes or simply by vigorously washing with phosphate buffered saline (PBS). Cells in the washes were transferred into a 6-well tissue culture plate and cultured for 12 hours to 24 days in a CO_2 incubator at 37° C. It should be noted that, in case of patients with high white blood cell count, i.e., patients with lymphoma or leukemia, the mononuclear cell fraction should be diluted with the complete medium to 1-2 million cells per ml for one well of 6-well tissue culture plate. Recovery of cancer cells is dependent on cell density on the matrix (see below). Viability of the cells was evaluated by Trypan Blue exclusion or apoptosis assays.

Alternatively, whole blood is deprived directly of viable and invasive carcinoma cells by passing through cell culture beads coated with a cell-adhesion matrix such as type I collagen or filters made of purified cotton. A sterile 30-ml pipette was packed with purified cotton or collagen-coated beads, and 0.1-ml of collagen-coated bead was used for every 10-ml blood that was pre-diluted with equal

volume of medium containing 0.5 mM EDTA or with 10% anticoagulant citrate dextrose (ACD, Bakter Healthcare Corporation, IL) containing 50 units heparin/mL. The column was pre-washed by medium containing 10% human plasma or serum. Flow rate, from 0.1 to 0.7 ml per minute, results in capture of cells that share similar features as these derived from the substratum method described above.

6.1.4 LABELING OF COLLAGEN MATRIX AND MEASUREMENTOF THE CELL INVASIVE PHENOTYPE

Collagen was polymerized prior to biotin, fluorescein or rhodamine labeling so that sites of polymerization were not perturbed. Labeled collagen fibers were then solubilized in acidified water (pH 2.0), but could be readily polymerized back to collagen fibers under experimental conditions. Specifically, 10 ml type I collagen solution (rat-tail type I collagen, 4 mg/ml, Collaborative Biomedical Products, Becton and Dickinson Labware, Bedford, MA) was mixed with 10-ml DMEM and added into a 10 centimeter tissue culture plate at 4° C. The plate was incubated for 30 min at 37° C to allow polymerization of the collagen fibers (gel). The gel was washed with 30 ml of coupling borate buffer, pH 9.3 (Sigma) for 30 min and then incubated with 30 ml borate buffer containing 3 mg of Sulfo-NSH-Biotin (Pierce), Fluorescein Isothiocyanate I hydrochloride (FITC), Tetramethyl Rhodamine Isothiocyanate (TRITC) (Research Organics Inc, Cleveland, OH) or 1 mg of Bodipy-rhodamine or fluorescein dyes (Molecular Probes, Inc.), at 25° C on a shaker. Conjugation was stopped by washing 3 times with PBS, followed by a 50-ml PBS washing for 2 days and a 50-ml distilled water wash for another 2 days. Labeled collagen fibers were solubilized in acidic water (0.02N acetic acid) to a final concentration of 1 mg/ml. Labeled collagen monomers were mixed with equal volume of unlabeled collagen solution, and further diluted with 2X volume of DMEM, overlaid on vessel surfaces to form a thin layer, and incubated for 30 min at 37° C to allow gel formation.

Labeled collagenous matrix was coated on a 16-well microtiter plate-glass slide (in 96-well microtiter plate format; Lab-Tek, Rochester, NY) or 6-well tissue culture plate (NUNC). A portion of mononuclear cells, i.e., 0.1-ml per well of 96-well microtiter plate or 1.6-ml per well of 6-well tissue culture plate

(NUNC), were seeded on such substratum and cultured for 15 minutes to 1 hour to capture adherent cells. After washing of non-adherent cells, the cells were grown on the labeled matrix for 12 to 24 hours for measurement of cellular invasiveness using Nikon Eclipse E300 inverted light microscope in conjunction with SONY DC5000 Cat Eye Imaging system or Molecular Devices fMax Fluorescence Microplate Reader in conjunction with SOFTmaxPRO 1.2F for Windows software computer analysis as depicted in Figure 1A , 1B. Initially, the Bodipy-fluorescent collagen substrata described above were prepared in 16-well culture chambers to optimize cell culture and microscopic imaging analysis conditions for characterization of the invasiveness of the matrix-isolated cells (Figure 1A). Substrate preparations were then optimized for 96-well microtiter plate version. Optimization parameters include feasibility for sample throughputs, and resolution for accuracy of quantitation.

6.1.5 OTHER CELLULAR FUNCTION ASSAYS

A functional assay was performed for endothelial cell activity using acetylated low-density lipoprotein (acLDL) Bodipy FL conjugate as described in manufacture manual (L-3485, Molecular Probes, OR, USA). To resolve blood cells co-isolated on the matrix that were apoptotic or necrotic, the cells were stained prior to fixation using Vybrant Apoptosis Assay Kit #5 Hoechst/prodidium iodide (V-13244, Molecular Probes, OR, USA).

6.1.6 CELL LINES AND CULTURE

The human breast carcinoma cell lines MDA-MB-436 and Hs578T were obtained from American Type Culture Collection (Rockville, MD), and the human amelanotic melanoma cell line LOX was obtained from Professor Oystein Fodstad, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway. Cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and RPMI1640 supplemented with 10% calf serum, 5% Nu-serum (Collaborative Research, Inc., Bedford, MA), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 1 unit/ml penicillin, and 10 μg/ml streptomycin. These cells were used to evaluate the reagents for immunocytochemical detection and to determine sensitivity of functional assays.

6.1.7 SAMPLE PREPARATION FOR IMMUNOCYTOCHEMISTRY

In order to enumerate transformed epithelial cells in the blood as compared to leukocytes and peripheral blood tissue cells, the mononuclear cells derived from blood of cancer patients that were captured by labeled collagenous matrix coated on a 16-well chambered glass slides (Lab-Tek, Rochester, NY) and cultured on the same substratum for 12-24 hours in a CO_2 incubator at 37° C were fixed for immunocytochemistry. Primary mAbs used in this study include mouse mAb recognizing human epithelial specific antigen (ESA; clone VU-1D9, NeoMarkers, CA, USA; SIGMA, MS, USA), Muc-1 epithelial membrane glycoprotein (Muc-1; clone E29, NeoMarkers, CA, USA), cytokeratins 4,5,6,8,10,13, and 18 (PCK; clone C-11, SIGMA, MS, USA); CD31/PECAM-1 endothelial cell marker (CD31; Clone JC/70A, NeoMarkers, CA, USA), Flk-1, a receptor for vascular endothelial growth factor (Flk-1, Clone sc-6251, Santa Cruz, USA), VE-cadherin endothelial marker (VE-cad; Clone sc 9989, Santa Cruz, USA); CD34 peripheral blood stem cell marker (CD34; clone 581, Pharmingen, USA), CD45 leukocyte common antigen (CD45; clone T29/33, DAKO, Denmark), CD8 suppressor T cell marker (CD8; clone c8/144B, NeoMarkers, CA, USA), CD43 T cell marker (CD43; clone 84-3C1, NeoMarkers, CA, USA), prostate specific acid phosphatase (PSAP; clone PASE/4LJ, NeoMarkers, CA, USA), prostate specific antigen (PSA; clone ER-PR8, NeoMarkers, CA, USA), c-erbB-2/Her-2/neu oncoprotein (erB-2; clone e2-4001 + 3B5, NeoMarkers, CA, USA), c-erbB-2 (Clone TAB250, Zymed, CA, USA), CA 19-9/sialyl Lewis GI tumor marker (CA19-9; clone 121 SLE, NeoMarkers, CA, USA), or p53 tumor suppressor protein (p53; clone DO-7 + BP53-12, NeoMarkers, CA, USA). In addition, fluorescein conjugated antibodies against Muc-1 epithelial cell marker (DAKO, Denmark) and fluorescein conjugates of goat antibodies against factor VIII endothelial marker (F8; Atlantic) were used to doubly stain carcinoma and endothelial cells, respectively, in addition to above primary antibodies against other cell markers. Furthermore, rat mAbs D28 (against seprase), E19, E26 (against DPPIV) and C27 (against β1 integrin) generated in our laboratory were used.

Antibody staining involves the addition of primary antibody and/or fluorescein-F8 or -Muc-1 to the slides after blocking nonspecific binding sites with 2% BSA for 30 min. The samples were incubated for 20 min at room temperature, washed twice in PBS for 5 min, and then exposed to secondary rabbit anti-mouse Ig (Z0259, Dako) for another 20 min. After two more washes, the samples were incubated with alkaline-phosphatase-anti-alkaline-phosphatase (APAAP) mouse Ig complexes for 15 min. Finally, the enzyme-substrate [NewFuchsin (Dako)] was added, resulting in the development of red precipitates. The data were recorded by using Nikon Eclipse E300 inverted light microscope in conjunction with SONY DC5000 Cat Eye Imaging system and were stored on a computer server for later reference.

6.1.8 SAMPLE PREPARATION FOR FLOW CYTOMETRIC ANALYSIS

In order to enumerate transformed epithelial cells in the blood that were captured by labeled collagenous matrix coated on a 6-well tissue plate, the mononuclear cells released from the matrix substratum were analyzed by flow cytometry following a manufacture's procedure. Similar to procedures involved in immunocytochemistry, the cells were determined for apoptosis or necrosis by staining prior to fixation using Vybrant Apoptosis Assay Kit #5 Hoechst/prodidium iodide (V-13244, Molecular Probes, OR, USA). Briefly, the mononuclear cells were stained in a solution containing fluorescein (FITC)-conjugated mouse mAb C11 against cytokeratins 4, 5, 6, 8, 10, 13, 18 (PCK; Sigma) or fluorescein-antibodies against Muc-1 (DAKO), phycoerythrin (PE)-conjugated anti-CD31 endothelial marker (CD31; Becton-Dickinson) and peridinin chlorophyll protein (PerCP)-labeled anti-CD45 (CD31; Becton-Dickinson) for 15 min. After incubation and washing, the collected cells were resuspended in 0.5 ml of a buffer and the sample was analyzed on a FACScan or FACSVantage flow cytometer (Becton Dickinson).

6.2 RESULTS

6.2.1 ISOLATION OF CANCER CELLS USING THE CELL-ADHESIONMATRIX: ROLE OF BLOOD BORNE CELL ADHESION MOLECULES

Table I shows the attachment of LOX human malignant melanoma cells and cancer cells isolated from a patient with head-neck cancer to various natural fiber surfaces in the presence of human plasma fibronectin, laminin and vitronectin, as well as human and bovine sera. Attachment of human cancer cells were assayed in 96-well plates (Nunc, Inc., Naperville, IL) coated with either various amounts of purified serum proteins (0.1 to 1 μg per well) or 10% serum and was performed as described (Nomizu et al., 1995). Cell attachment properties of natural fibers were determined by counting the number of cells in the area that remained attached on 1.27 mm^2 areas of a microtiter well. In the presence of human or bovine sera or fibronectin + laminin + vitronectin, LOX melanoma cells adhere better on purified collagen, fibrin, and cotton fibers, as well as lined plastic surfaces than plain plastic, and have higher numbers of cells that remain attached (Table 1). Similarly, head-neck cancer cells attach better on purified collagen, fibrin, and cotton fibers, as well as lined plastic surfaces than plain plastic, and have higher numbers of cells that remain attached (Table 2). These results suggest that the surfaces of the cell-adhesion matrix exert their effects on cancer cell adhesion through their binding affinity for blood-borne adhesion molecules.

Table 1. Attachment of LOX melanoma cells on various cell-adhesion matrix surfaces is mediated primarily by binding of the matrix to blood-borne cell adhesion molecules.

	Human serum	Bovine serum	FN+LM+VN	Negative control
Type I collagen	92±5	91±6	86±5	8±5
Fibrin	68±7	72±8	67±6	6±6
Purified cotton	79±5	83±9	72±6	7±6
Lined plastic	57±8	49±9	35±8	4±3
Plain plastic	7±3	5±4	3±2	2±1

Cells, $4x10^3$ per well (the number of cells in the area that remained attached on 1.27 mm^2 areas of a microtiter well was counted), were seeded on various matrix surfaces in 96-well plates (Nunc, Inc., Naperville, IL), that were coated with 10% human serum (Sigma), 10% bovine serum (Collaborative Research, Inc., Bedford, MA), or human plasma fibronectin + laminin + vitronectin (FN+LM+VN; Gibco-BRL, Gaithersburg, MD) at the concentration of 10 μg/ml each. The wells that were not coated with protein were used as negative control. Type I collagen fibers were formed on the bottom of microtiter wells according to the procedure described below. Fibrin fibers were made by clotting 20% human plasma in Dulbecco's modified Eagle's medium in microtiter wells. Purified cotton fibers were also suspended in Dulbecco's modified Eagle's medium and seeded in microtiter wells. "Lined plastic" indicates that the surface of microtiter wells had been scrapped with an Eppendorf peptide tip. Each value represents the mean ± S.D. of three independent experiments.

Table 2. Attachment of head-neck cancer cells on various cell-adhesion matrix surfaces is mediated primarily by binding of the matrix to blood-borne cell adhesion molecules.

	Human serum	Bovine serum	FN+LM+VN	Negative control
Type I collagen	42±4	41±4	37±5	2±2
Fibrin	37±3	33±5	27±3	3±2
Purified cotton	38±5	34±4	29±2	2±1
Lined plastic	27±2	28±3	22±2	2±1
Plain plastic	5±2	3±2	2±1	2±1

Cells, $2x10^3$ per well (the number of cells in the area that remained attached on 1.27 mm^2 areas of a microtiter well was counted), were seeded on various matrix surfaces in 96-well plates (Nunc, Inc., Naperville, IL), that were coated with 10% human serum (Sigma), 10% bovine serum (Collaborative Research, Inc., Bedford, MA), or human plasma fibronectin + laminin + vitronectin (FN+LM+VN; Gibco-BRL, Gaithersburg, MD) at the concentration of 10 μg/ml each. The wells that were not coated with protein were used as negative control. Type I collagen fibers were formed on the bottom of microtiter wells according to the procedure described below. Fibrin fibers were made by clotting 20% human plasma in Dulbecco's modified Eagle's medium in microtiter wells. Purified cotton fibers were also suspended in Dulbecco's modified Eagle's medium and seeded in microtiter wells. "Lined plastic" indicates that the surface of microtiter wells had been scrapped with an Eppendorf peptide tip. Each value represents the mean ± S.D. of three independent experiments.

6.2.2 SENSITIVITY OF CELL SEPARATION METHODS

The human invasive breast carcinoma cell lines MDA-MB-436 and Hs578T (American Type Culture Collection, Rockville, MD) were used to determine sensitivity of the cell separation method and to evaluate the reagents for immunocytochemical detection (see below). These breast cancer cells were tagged

with a fluorescent dye to determine the sensitivity of the isolation procedure. Figure 3A–G show the analysis of normal and bladder cancer blood samples to which varying numbers of Hs578T breast carcinoma cells, which were tagged with PKH26 Red Fluorescent Cell Linker (Sigma), were added. As can be seen, the carcinoma cells can be distinguished from the other blood cells. The recovery of breast carcinoma cells was consistent over a frequency range, between 1 and 500 Hs578T cells spiked into 1-ml of blood (10 – 20 million mononuclear cells per ml) from normal donors, and the recovery rate was between 75 and 100%. However, when Hs578T breast carcinoma cells were spiked into the blood from a bladder cancer donor with less than 10 million mononuclear cells per ml, the recovery of the Hs578T cells was between 95 and 100%, suggesting cell density is a limiting factor for cell adhesion to the matrix. In addition, when 19 red fluorescent tagged Hs578T cells were spiked into 1-ml of blood from a bladder cancer donor, both isolated Hs578T and bladder cancer cells were identified by positive staining with anti-cytokeratin antibody C11 (Figure 3E - 3F). There were 16 fluorescent tagged Hs578T cells and 468 bladder carcinoma cells detected in one ml blood. These results suggest that the level of sensitivity by the matrix capture method is at 1 viable cancer cell per ml of blood, and the recovery rate can reach 85%.

6.2.3 CANCER CELL ISOLATION USING THE CELL-ADHESION MATRIX

For detection of cancer cells in blood, a thin layer of rhodamine-collagen solution coated on 16-well chambered glass slides (Lab-Tek, Rochester, NY) or 96-well culture plates (NUNC), which is subsequently incubated with calf serum containing blood-borne adhesion molecules, is a preferred embodiment of the "matrix". Mononuclear cells in buffy coat derived from 1.6 millimeter (ml) of whole blood are seeded in equal aliquots, 0.1 ml each, into each well of a 16-well chambered glass slide, and incubated for 10 minutes to 18 hours at 37° C to select for cancer cells by differential adhesion to the collagen matrix.

Figure 4A-D depict the isolation of circulating cancer clusters with tissue fragments using such a type I collagen based, cell-adhesion matrix. Such cell clusters of various sizes were commonly observed in the blood of patients with advanced metastatic disease, including squamous cell carcinoma of head and neck

(Figure 4A-C) and prostate cancer (Figure 4D). It has been estimated that there are large numbers of cancer cell clusters in the blood of stage III-IV cancer patients, ranging from a few hundred to twenty thousand per cubic centimeter of blood. However, there are no such cell clusters found in the blood of normal donors or early stage cancer patients. Furthermore, fibrin fibers (Figure 4E), plastic scraps (Figure 4F-G), purified cotton fibers (Figure 4H-I), and type I collagen fibers (Figure 5A-H) capture circulating carcinoma cells that grow in culture and exhibit epithelial cell shape. Surfaces of these materials absorb blood-borne cell adhesion molecules, which in turn promote the attachment and spreading of tumor cells (see Table II above). The role of cell adhesion molecules in this process has been further demonstrated by incubation of coated collagen matrix with polyclonal antibodies directed against fibronectin, vitronectin, and laminin. This treatment resulted in a reduction of cell capture capability of the matrix.

Figure 5A-H show the morphology of circulating cells and their underlying type I collagen-based, cell-adhesion matrix in the presence of blood-borne cell adhesion molecules. These carcinoma cells adhere preferentially to the collagen matrix within 10 minutes to 60 minutes after contact. They then degrade and ingest the matrix generating holes on flat substrata, followed by migration and growth on the plastic surface (Figure 5A-D; 6A-D). Such carcinoma cells are not present in blood of most normal donors (Figure 7A), patients with benign disease (Figure 7B), or cancer patients undergoing chemotherapy (Figure 7C-D). Freshly isolated circulating cancer cells are small in size but relatively larger than most of mononuclear blood cells co-isolated on the matrix (Figure 6A). These putative cancer cells grow rapidly, become larger in size, and assume epithelial shape within four days in culture (Figure 5A-D; 6A-D). However, co-isolated leukocytes are small and reduce their number in culture (Figure 5A-D). Also, few carcinoma cells isolated from squamous cell carcinoma of head and neck biopsies (SCCHN) using the cell-adhesion matrix (Figure 5E-F) exhibited the invasive phenotype or growth characteristics of circulating cancer cells, suggesting that circulating cancer cells represent a unique subset of carcinoma cell population that metastasizes. In such SCCHN tissue, they are fibroblasts that invade the collagen gel and propagate in culture (Figure 5E-F). It should be noted

that the cell separation system captures cells which remain viable, i.e., cells which retain the ability to adhere, but not those that are damaged in the circulation or by experimental manipulation.

The thickness of the collagen matrix was estimated approximately 100 μm, as determined by both light microscopic and transmission electron microscopic measurements (Figure 5E-H). The assembled collagen can capture cancer cells from mixed cell population, i.e., blood or cell suspension derived from tissue biopsy, within 10 minutes to one hour. The matrix-captured cells can further invade into the matrix during the one to two week incubation, leaving behind some non-invasive cells, which do not multiple, on the surface of the matrix (Figure 5E-F). The assembled collagen fibers are fine strings of 70 nm in diameter and have similar appearance as these in the collagen bundles *in vivo* except they lack the collagen-banding pattern (Figure 5G-H).

6.2.4 FUNCTIONAL AND IMMUNOPHENOTYPIC FEATURES OF CIRCULATING CANCER CELLS

The functional and immunophenotypic features of the epithelial cells captured by the matrix are consistent with those of neoplastic cells. For measurement of the invasive phenotype of these cells, cells that were isolated from blood of cancer patients using rhodamine-collagen matrix were analyzed for the ability of the cell to adhere to, degrade and ingest rhodamine-collagen substratum. Figure 8A-I show that putative carcinoma cells exhibit extensive collagen-degrading/ingestion activities (Col+); these cells also exhibit immuno- and morphological features characteristic of carcinoma cells (see below).

For the determination of nature of cancer cells, cells that were isolated from blood of cancer patients using rhodamine-collagen matrix were analyzed for their potential epithelial origin by immunocytochemistry using antibodies against epithelial specific antigen (ESA), epithelial membrane antigen (Muc-1), and cytokeratins 4,5,6,8,10,13, and 18 (PCK). These cells react positively with ESA+, Muc-1+, or PCK+ are epithelial origin, and therefore, consistent with their metastatic nature. On the matrix, adherent cells including enriched cancer cells are stained with cell type-specific monoclonal antibodies such as these against epithelial, carcinoma, endothelial, peripheral blood stem cells, or leukocytes. Adherent cells, that are

labeled positively for epithelial cell markers (Figure 8E, G, J, K) and that ingested collagen fragments, Col+ (Figure 8B, C, F, I, L), are identified as carcinoma cells. Circulating carcinoma cells are rare in blood of most normal donors (Figure 8L), patients with benign disease, or cancer patients undergoing chemotherapy. Similar to immunocytochemical analysis presented here, mononuclear cells derived from blood of cancer patients that were released from the matrix can be subject to fluorescence-activated cell sorting (FACS) analysis.

6.2.5 THE ACQUISITION OF AN ENDOTHELIAL CELL PHENOTYPE BY CIRCULATING CARCINOMA CELLS

In addition to invasion, metastatic carcinoma cells in the circulation involve an apparently novel angiogenic propensity. Circulating carcinoma cells isolated from the blood of cancer patients using a cell-adhesion matrix inevitably acquire endothelial characteristics. These squamous cell carcinoma of head-neck (HN, Figure 9A-C; 9I-J), colon cancer (CC, Figure 9D-E) and prostate cancer (PC, Figure 9G-H) cells are Col+ (Figure 9C, F), stain for the endothelial cell markers, including von Willebrand factor or factor VIII (F8) (Figure 9B, E) and CD31 (Figure 9D), and express an endothelial cell ability by incorporating acetylated low-density lipoprotein (LDL) (Figure 9H, J) as well as an epithelial surface antigen (ESA in Figure 9G). In addition, when the isolated carcinoma cells internalized and were labeled with fluorescein-LDL and then plated on a collagen gel of 0.5-mm in thickness, they showed enhanced endothelial differentiation, including the formation of cellular networks and tube-like structures (Figure 9K, L). These structures consisted principally of LDL-labeled carcinoma cells. Furthermore, circulating carcinoma cells illustrate additional endothelial features including the expression of the receptor for vascular endothelial growth factor Flk-1 and VE-cadherin, and without loss of previously expressed epithelial markers. This angiogenic propensity is restricted to those carcinoma cells that enter the circulation and not with cells that remain in the tumor tissue. Such spatial restriction in angiogenic phenotype reflects distinct functional abilities of circulating carcinoma cells to extravasate, colonize and cooperate with angiogenesis that lead to formation of micrometastases.

6.2.6 IMMUNE CANCER KILLING AND GROWTH OF CIRCULATING CANCER CELLS IN CULTURE

Circulating cancer cells form clusters with white blood cells (WBC) that express the leukocyte common antigens CD45 and the T-cell marker CD8. The cell-adhesion matrix could readily isolate such clusters of immune and cancer cell complex from cancer patients who exhibited encouraging prognosis. Although total number of circulating cancer cells may indicates a potential for metastasises formation, numerations of the cancer cells that interact with cytotoxic WBC (Figure 10A-I) or autoimmune anti-cancer antibody-mediated, complement cytolysis (Figure 10J-L) signify the host immunity against metastasis. In these cancer patients who exhibited favorable prognosis, most cancer cells were attacked by leukocytes and became fragmented after one day in culture and only a few cancer colonies grew (Figure 10F-I). In addition, anti-cancer antibody-mediated complement cytolysis can take place in most cancer patients. Such killing of cancer cells was demonstrated here by culturing isolated BLC cells in the presence of 10% autologous plasma, derived from the blood of the same cancer patient (Figure 10J-K), but not in those from a normal donor (Figure 10L). Resulting cytolysis of cancer cells can be determined by morphological appearance of phase-dark materials in lysed cells (Figure 10J-L). Importantly, approximately 97% cells freshly isolated from the blood are apoptotic as determined by fluorescent staining using apoptosis and cell lyses kits produced by Molecular Probes, Inc. Although some cancer cells can propagate in the culture for two months, most cancer cells show apoptosis or cytolysis immediately after their isolation, indicating cancer cell killing by host immunity. Most WBC disappear from the culture after one week, leaving behind clusters or colonies of cancer cells (Figure 10F-L). Thus, numeration of viable and invasive cancer cells that resist immune killing would be the strongest indicator for patients who process high degree of malignancy.

6.2.7 ENUMERATION OF CIRCULATING CANCER CELLS

Circulating cancer cells isolated using the cell separation methods were analyzed for their invasive activities, epithelial nature, angiogenic propensity, and resistance to host immunity as described above. Results were recorded using an

inverted Nikon microscope computerized with a SONY DKC5000 3CCD imaging system. Numeration of cancer cells present in whole blood or buffy coat by this cell separation and assay methods described herein presents the highest sensitivity and resolution. Over 200 blood samples of patients with prostate, breast, colon, lung, head & neck, brain, bladder, lymphoma, renal & testis, liver, or pancreatic and other gastrointestinal cancers were examined. Circulating cancer cells, as defined by Col+, LDL+, ESA+, Muc-1+, PCK+, F8+, or CD31+ cells, are in the order of two thousand to twenty thousand per ml of whole blood derived from cancer patients with metastatic diseases. From this study, it is estimated that there are 8 to 80 million viable cancer cells in the circulation of a cancer patient which have the potential to metastasize. This is over 100-fold higher resolution than previous antibody-based approach (Racila,E., Euhus,D., Weiss,A.J., Rao,C., McConnell,J., Terstappen,L.W., and Uhr,J.W., 1998, Detection and Characterization of Carcinoma Cells in the Blood. Proceedings of the National Academy of Sciences of the United States of America 95, 4589-4594). However, the number of metastatic cells estimated from this study represents only 0.1% of total circulating cancer cells as reported in a previous investigation (Glaves et al., 1988, Br. J. Cancer 57:32-35).

7. EXAMPLE: REGULATION OF FIBROBLAST MIGRATION ON A COLLAGENOUS MATRIX IS DEPENDENTON A NOVEL CELL SURFACE PROTEASE COMPLEX

The data provided below, demonstrates that the formation of a novel protease complex, consisting of serine integral membrane proteases (SIMP), including seprase and dipeptidgl peptidase IV (DPPIV), at invadopodia of migrating fibroblasts is associated with cell invasion and migration on a collagenous matrix.

7.1 MATERIALS AND METHODS

7.1.1 CELL CULTURE

The human embryonic lung fibroblastic line WI38 and human breast carcinoma cell line MDA-MB-436 were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in a 1:1 mixture of Dulbecco's

modified Eagle's medium (DMEM) and RPMI1640 supplemented with 10% calf serum, 5% Nu-serum (Collaborative Research, Inc., Bedford, MA), 2 mM L-glutamine, 1 unit/ml penicillin, 10 g/ml streptomycin, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. Rat mAbs E26, E19 and F4 are directed against human placental DPPIV, rat mAbs D8 and D28 are against human placental seprase (Goldstein LA et al., 1997, *Biochim. Biophys. Acta.* 1361:11-19; Pinereiro-Sanchez, ML et al., 1997, *J. Biol. Chem.* 272: 7595-7601; correction (1998) *J. Biol. Chem.* 272:13366), mAb C27 is directed against human melanoma β1 integrin and rat mAb C37 is directed against cell surface glycoprotein gp-90 (Meuller, SC., et al., 1999, *J.Biol. Chem.* 274:24947-24952). Mouse anti-αv integrin and anti-β3 integrin mAbs were from American Type Culture Collection (clone L230, catalog number HB8448 and clone AP-3, catalog number HB242, respectively).

7.1.2 ISOLATION OF SEPRASE-DPPIV COMPLEX

WI38 cells were seeded onto hydrated type I collagen films (rat tail type I collagen at 0.2 mg/ml, Collaborative Biomedical Products, Becton and Dickinson Labware, Bedford, MA) and cultured until 90% confluence. Surface biotinylation of WI38 monolayers were performed using Suffo-NSH-Biotin (Pierce, Rockford, IL) according to manufacturer's instructions. To harvest lysates, each culture plate was washed 3 times with PBS and extracted with 125 l/cm^2 of RIPA solution (1.25% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in 50 mM Tris buffer, pH 7.5). Extraction was performed for 2 h at 25°C, 25 rpm on a rotary shaker (Bellco Orbital Shaker, Vineland, NJ). The cell layer and buffer were removed and transferred to a 50 ml conical tube and incubated a further 3 h at 4°C with end-over-end agitation. The extract was clarified by centrifugation at 10,000 x g for 20 min at 4°C and the supernatants used for immunoprecipitation reactions.

To prepare immunoaffinity matrix, purified rat mAbs against membrane proteins (2.5 mg) were coupled to 1-ml CNBr-Sepharose 4 MB (Pharmacal Biotech Inc., Piscataway, NJ). 0.25-ml mAb-beads were used to immunoprecipitate complexes from 25-ml cell extract overnight at 4°C with end-over-end agitation. After 3 washes in 25 ml of extraction buffer, the beads with coupled antibody-antigen complexes were resuspended in 0.1 % glycine-HCl (pH 2.4) buffer (equal to the bead

volume) and the sample was incubated for 5 min at 4°C. Immediately, the sample was transferred to an Amicon filter insert (0.45 m 400 l c apacity) and centrifuged 10 min at 10,000 rpm in an Eppendorf microfuge at 4°C. The bead filtrate was neutralized by addition of 2M Trizma base. To determine the subunit composition of isolated protein complexes, immunoprecipitates of the surface-biotinylated complexes were analyzed for their protein profiles by SDS-PAGE, transferred to nitrocellulose membranes and detected with HPR-streptavidin (Sigma, St. Louis, MO) and the ECL system (Amersham, St. Louis, MO). Isolated protein complexes were also used for immunoblotting (with anti-seprase, -DPPIV, -β1 and -β3 integrin mAbs), for gelatin zymography (to detect seprase gelatinase activity), and for DPPIV substrate membrane overlay as described (Pinereiro-Sanchez, ML et al., 1997, *J. Biol. Chem.* 272: 7595-7601; correction (1998) *J. Biol. Chem.* 272:13366). To test for complete elution of proteins from the beads, Laemmli sample buffer (equal to the bead volume) was added and samples were heated by microwaves (2 cycles on low setting 30 sec each, followed by 1 cycle on medium for 30 sec). Then, the samples were immediately centrifuged at 4°C. The filtrates were subjected to assays described above.

7.1.3 LABELING OF COLLAGEN FIBERS

Collagen was polymerized prior to biotin, fluorescein or rhodamine labeling so that sites of polymerization were not perturbed. Labeled collagen fibers were then solubilized in acidified water (pH 2.0), but could be readily polymerized back to collagen fibers under experimental conditions. Specifically, 10 ml type I collagen solution (rat-tail type I collagen, 4.66 mg/ml, Collaborative Biomedical Products, Becton and Dickinson Labware, Bedford, MA) was mixed with 10-ml DMEM at 4°C. The mixture was incubated for 30 min at 37°C to allow polymerization of the collagen fibers (gel). The gel was washed with 30-ml of coupling borate buffer, pH 9.3 (Sigma) for 30 min and then incubated with 30-ml borate buffer containing 3 mg of Sulfo-NSH-Biotin (Pierce), Fluorescein Isothiocyanate I hydrochloride (FITC) or Tetramethyl Rhodamine Isothiocyanate (TRITC) (Research Organics Inc, Cleveland, OH) at 25°C on a shaker. Conjugation was stopped by washing 3 times with PBS, followed by a 50-ml PBS washing for 2

days and a 50-ml distilled water wash for another 2 days. Labeled collagen fibers were solubilized in acidic water (0.02N acetic acid) to a final concentration of 1 mg/ml. Labeled collagen monomers were mixed with equal volume of DMEM or p-buffer (300 mM NaCl in 50 mM ammonium bicarbonate buffer, pH 8.4) and incubated for 30 min at 37°C to allow gel formation.

7.1.4 CELL MIGRATION AND FLUORESCENT COLLAGEN DEGRADATION ASSAYS

Fluorescent collagen fibers overlaying a monolayer wound culture were used to examine cell migration in collagen gel during wound closure. WI38 cells were grown in 2-well chambered cover slips (Lab-Tek, Rochester, NY) to confluence. The monolayer was scratched with a pipette tip to generate wound edges. Culture media were then replaced with TRITC-collagen in DMEM (600 g/ml ; 50 l/well) and the culture allowed to gel in a CO_2 incubator for 30 min at 37°C. Media containing serum or inhibitory mAbs (300 1 /well) were then added and their effects on cell migration and collagen degradation in real time were observed using phase contrast and fluorescence microscopy (Nikon Inverted Microscope). Cell migration and collagen degradation were quantified by measuring the areas of cell migration and fluorescent collagen removal by migratory cells using NIH Image 1.62b4/fat analysis program.

A microtiter version of the above assay was developed to measure collagen degradation by migratory cells. In a 96-well tissue culture plate (Nunc, Rochester, NY), 50 l/well of TRITC-collagen solution (600 g/m l) was first loaded and the solution allowed to gel in 37°C incubator for 30 min. The TRITC-collagen gel was then overlaid with 50 l/well of the TRITC-collagen solution containing 2×10^5 cells and mAbs (50 g/ml), and the culture allowed to gel in a CO_2 incubator for 30 min at 37°C. The culture was then supplemented with 150 l fresh media per well. All media were prepared free of phenol red. At times 100 l of culture media from each well was removed to measure the release of TRITC-collagen peptides using a fluorescent microplate reader with excitation at 544 nm and emission at 590 nm (Molecular Devices fMax: Fluorescence Microplate Reader). Leucine incorporation was used to determine metabolic activities of cells in culture conditions, in which

150 l/well of media containing 2 Ci/ml ^{3}H-Leucine was added into the culture, and the cell-collagen layers were solubilized in 5 ml of scintillation fluid and counted in a scintillation counter (Beckman LS-7500).

Stable transfectants of the breast carcinoma line MDA-MB-436 that expresses constitutively seprase and DPPIV were obtained. Plasmid pA11 (pCR3.1 vector alone), pA15 (vector plus full length seprase), and pZ8 (DPPIV ribozyme construct) were transfected into MDA-MB-436 cells using lipofectamine (Gibco/BRL) following the manufacture's instructions. The selection medium contained G418 at a concentration of 300 g/ml.

7.1.5 IMMUNOFLUORESCENT LABELING OF SEPRASE AND DPPIV

WI38 cells were cultured in collagen gel, fixed and immunolabeled in a single step using rhodamine conjugated mAbD28 against seprase and fluorescence conjugated mAb E26 against DPPIV as described (Meuller, SC, et al., 1999, *J. Biol. Chem.* 274:24947-24952). Stained samples were photographed using the Planapo 25/1.2 or 63/1.4 objectives on a Zeiss Photomicroscope III (Carl Zeiss, Inc.) under epifluorescence.

7.1.6 HUMAN GINGIVAL WOUNDS AND INVASIVE HUMAN BREAST CARCINOMA

Human gingival biopsies were derived from the University of Turku, Finland. Full thickness wounds of oral mucosa were made from two healthy volunteers and biopsies were collected after 3, 7, 14 and 28 days of wounding. Immediately after biopsy, fresh tissue blocks were mounted in Histoprep® (Fisher Scientific, New Jersey) and snap frozen in liquid nitrogen. Frozen sections (6 m) were cut, fixed with -20°C acetone for 5 min, and stored at -70°C. For routine histology, the sections were stained with hematoxylin and eosin. For immunohistochemical staining, sections were washed with PBS containing 0.1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, M.O.) and incubated with rhodamine-conjugated mAb D28 against seprase in PBS/BSA in humid chamber at 4°C for 16 h. The sections were then washed with PBS/BSA and water, briefly air-dried, and mounted using cyanoacrylate glue (Krazy Glue, Borden Company LTD).

The staining was examined using a Zeiss Axioskop 20 light, fluorescence and confocal microscopy, and photographed using MC 80 Zeiss microscope camera. Control staining was performed with rhodamine-conjugated secondary antibody and showed no specific stain. Immunohistochemical staining of invasive human breast carcinoma was performed as described (Kelly T et al., 1998, *Mod. Pathol.* 11:855-863).

7.2 RESULTS

The data presented indicates that like seprase in LOX human malignant melanoma cells (Pineiro-Snachez ML, 1997, *J. Biol. Chem.* 272:7595-7601), the majority of seprase and DPPIV in W138 human embryonic fibroblasts were present as a >400-kDa complex in non-ionic detergents, including Triton X-100, Triton X-114, RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS), and octyl glucoside, and in WGA-agarose affinity-purified material. The >400-kDa complex eluted in the void volume fractions on Sephacryl S-200 gel filtration chromatography. Isolation of WGA-purified material followed by Superose 12 gel filtration liquid chromatography, exhibited major forms of about 200-kDa (Fraction 17), 440-kDA (Fraction 14), and 670-kDa (Fraction 13) (Figure 11A). As seprase contains a 97-kDa subunit and DPPIV a 110-kDa monomer and as both seprase and DPPIV are dimers in non-ionic detergents (10), the gel filtration data suggests the presence of the seprase-DPPIV complex at 440-670 kDa sizes (Figure 11B).

Immunoprecipitation using mAb D28 (against seprase) and mAb E19 (against DPPIV) identified two major similar intensity bands in the WI38 cell extract that was surface-biotinylated (Figure 12A). The two bands, cross-immunoprecipitated by mAb D28 or E19, indicate seprase and DPPIV dimers, respectively. In such SDS gels when samples were solubilized in 1% SDS and not boiled, the top or slower band at 200-kDa was identified by immunoblotting as DPPIV, and the lower or faster band migrating at 170-kDa as seprase, respectively (Figure 12B). The 350-400 kDa heteromeric aggregate was not detected in SDS gels following SDS solubilization of the sample (Figure 12A, 12B), suggesting that the heteromeric aggregate dissociated into two stable dimers of 200-kDa DPPIV and 170-kDa seprase, respectively. In three independent experiments involving RIPA cell extracts, a stable association of

seprase and DPPIV was detected using mAbs against seprase and DPPIV but not those against β1 and β3 integrins (Figure 12A, 12B). Furthermore, such heteromeric complex was demonstrated by the proteolytic activities of the immuno-isolated complex. Antigens were isolated from WI38 RIPA extracts by affinity purification using either mAb D28 or mAb E19 that recognize seprase or DPPIV, respectively. The eluates were analyzed for a 170-kDa (seprase) gelatinase (Figure 12C) and 200-kDa (DPPIV) proline-specific dipeptidyl-aminopeptidase (Figure 12D). Gelatin zymography detected a 170-kDa gelatinase activity in immunoprecipitates of anti-seprase mAb D28 (Figure 12C, IP: seprase) or anti-DPPIV mAb E19 (Figure 12C, IP: DPPIV). Isolated DPPIV dimer has no gelatinase activity and the 170-kDa band on the gelatin zymogram identified with the DPPIV antibody represents the presence of seprase in the complex. Similarly, DPPIV substrate overlay assay detected a 200-kDa proline-specific dipeptidyl-aminopeptidase activity in immunoprecipitates of anti-seprase mAb D28 (Figure 12D, IP: seprase) or anti-DPPIV mAb E19 (Figure 12D, IP: DPPIV). No 170-kDa gelatinase of DPPIV activities could be observed for αv, α2, α6 or β3 integrin or control immunoprecipitates. These results also confirm previous observations that seprase and DPPIV are homodimers in SDS buffer, which are sensitive to heat (>60°C) and acid for dissociation into their monomeric subunits. Thus it appears that the protease complex contained equal amounts of seprase and DPPIV proteins (Figure. 11A, 11B, 12A, 12B), and it was equally effective in eliciting 170-kDa gelatinase and 200-kDa DPPIV dieptidyl-aminopeptidase activities (Figure 12C, 12D).

To determine the role of the seprase-DPPIV complex in cell migration in collagen gel and possible collagen degradation we overlaid a thin layer of collagen on a cell monolayer wound model for morphological examination and on a sparse culture for biochemical study (Figure 13). Cell migration in collagen gel and local collagen removal by cells were measured by counting the area of cell migration/collagen removal using image analysis in conjunction with phase contrast and fluorescence microscopy (Figure 13A-13D). Addition of mAb E19 (against DPPIV) into the wound-closure model blocked cell migration (Figure 13B, 13C) and local collagen removal by cells (Figure 13D), while that of a class matched mAb

(IgG) did not (Figure 13B, 13C, 13D). There was an increase in inhibition with increasing amounts of mAb E19 (Figure 13B) and the antibody inhibitory effect could be reversed by removal of E19, thus, this mAb was not toxic (Figure 13C). Furthermore, local collagen degradation by activated cells was quantified by counting fluorescent peptides released from fluorescent collagen fibers by W138 cells in a sparse culture in a 96-well plate using spectrofluorometry (Figure 13E). Cells in sparse culture are known to be migratory due to less "contact inhibition of migration" (Chen, W-T, 1979, J. *Cell Biol* 81:684-691). Migratory W138 cells showed time-dependent collagen degradation within 4 days, and mAb E19 (against DPPIV) inhibited collagen degradation by migratory cells while the control mAb C37 (anti-gp-90) did not (Figure 13E). These data demonstrate the role of the seprase-DPPIV complex in cellular migration in collagen gel and in the collagen degradation by wound-activated fibroblasts.

As DPPIV was shown to be an adhesion receptor for collagen (Bauvois B, 1988, *Biochem. J.* 252:723-731; Hanski C, 1988, *Exp.Cell Res.* 178:64-72; Loster K., 1995, *Biochem. Biophys. Res. Commun.* 217:341-348) or fibronectin (Cheng, HC 1998, *J. Biol. Chem.* 272:24207-24215; Johnson RC et al., 1993, *J. Cell Biol* 121:1423-1432; Piazza, GA et al., 1989, *Biochem. J.* 262:327-334)), it was determined whether the inhibitory effect of mAb E19 (against DPPIV) on cellular migration in collagen gel and on the collagen degradation by migratory cells is due to its influence on adhesion activity. Figure 14 shows that, in parallel comparison to integrin adhesion to collagen fibers , while mAb E19 (against DPPIV) inhibits cellular migration in collagen gel and on the collagen degradation by migratory cells it does not affect WI38 cell spreading on collagen substratum (Figure 14A) and attachment to collagen substratum (Figure 14B). However, mAb C27 (against β1 integrins) inhibits WI38 cell spreading on and adhesion to collagen substratum but mAb E19 (against DPPIV) or mAb C37 (anti-gp-90) do not (Figure 14A and 14B). These indicate that β1 integrins may be primary collagen receptors on WI38 cells responsible for substrate binding of the seprase-DPPIV complex.

To demonstrate that seprase and DPPIV are associated in invadapodia of migratory cells, double label, immunofluorescence experiments were performed

(Figure 15). It was observed that invadapodia of the cell migrating in collagen gel (a) were stained positively with TRITC-mAb D28 against seprase (b) or FITC-mAB E26 against DPPIV (c). Superimposed image also shows that seprase and DDPIV co-localize at the invadapodia of a WI38 fibroblast migrating in collagen gel (d).

To examine if such seprase and DPPIV co-localization could be found *in vivo* immunohistochemical experiments were performed on serial sections of formaldehyde-fixed, paraffin-embedded, human breast carcinoma tissue (Figure 16 and 18). Like tumor cells in the invasion front, connective tissue cells in human invasive breast carcinoma were strongly reactive with mAb D28 against seprase or mAB E26 against DDPIV (Figure 16, arrows). However, such seprase and DPPIV staining was not detected in connective tissue cells of distant normal tissues (Figure 18). It is likely that seprase-DDPIV expression associates with the activation of connective tissue cells in response to cancer invasion; and seprase and DPPIV co-localize in these activated tissue cells as well as in invasive carcinoma cells.

Unlike human umbilical cord smooth muscle cells in culture (Goldstein LA et al., 1997, *Biochim. Biophys. Acta.* 1361:11-19), both seprase and DPPIV preferentially distribute among mesenchymal cells but not differentiated muscle and endothelial cells of large vessels in human embryonic tissues, including placenta and umbilical cord. To determine if seprase and DDPIV expression in stromal fibrobalsts is induced during wound closure in vivo, the immunohistochemistry of human gingicval mucosa-wound closure was investigated (Figure 17). A strong expression of seprase and DDPIV was seen in connective tissue cells at day 3 after wounding (b-d, g). No immuno-reaction was seen in adjacent normal mucosa tissue. No specific reaction was seen in the fibrin clot area and epithelium. Confocal microscopy shows the localization of seprase (d) and DPPIV in protrusions of connective tissue cells, which indicate the existence of invadapodia in vivo. Later, at day 7 after wounding, only a few cells in the middle of granulation tissue were reactive with the anti-seprase antibody (f) but not the anti-DPPIV antibody (h). Seprase and DDPIV staining disappeared from connective tissue cells after one week and cells of 14 or 28-day-old wounds also did not react with the antibody. The data indicates that seprase and DDPIV are activation enzymes on

fibroblastic, endothelial and carcinoma cells, and that they may participate in the local collagen degradation necessary for cellular migration.

Cell transfection experiments were used to explore the cell surface association of seprase and DPPIV. MDA-MB-436 breast carcinoma cells that express constitutively the seprase-DPPIV complex were used. Cells over-expressing seprase or defective in DPPIV or seprase production were examined for their collagen-degrading and migratory activities in collagen gels (Figure 19). An increase in release of TRITC-collagen peptides was observed in MDA-MB-436 cells transfected with plasmid pA15 that encodes seprase (Figure 19A, pA15) and a decrease of peptide release was seen in cells transfected with a construct that encodes a DPPIV specific ribozyme (Figure 19A, pZ8) as compared to parental or vector transfected cells (Figure 19A; Parent, pA11). Also, over-expression of seprase appears to associate with a slight reduction of DPPIV, and cells transfected with the DPPIV ribozyme produce no detectable DPPIV and substantially reduced seprase (Figure 19B).

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims. Various publications are cited herein that are hereby incorporated by reference in their entireties.

CLAIMS

1. A method for detecting the presence of cancer cells in a subject comprising:

(a) inoculating a sample derived from said subject onto a matrix wherein said matrix is coated with adhesion molecules;

(b) incubating the sample for a time sufficient to allow adhesion of cancer cells to the cell-adhesion matrix; and

(c) detecting the presence of cancer cells bound to the matrix,

2. The method of claim 1 wherein the sample is a blood sample.

3. The method of claim 1 wherein the sample is a tissue sample.

4. The method of claim 1 wherein the matrix comprises a natural material, including collagen, fibrin and cotton fibers.

5. A method for determining the metastatic potential of cancer cells derived from a cancer subject comprising:

(a) inoculating the cancer cells derived from the cancer subject onto a matrix wherein said matrix is coated with adhesion molecules;

(b) incubating the cancer cells for a time sufficient to allow adhesion, ingestion or invasion of the matrix by the cancer cells to occur; and

(c) detecting adherence, ingestion or invasion of the matrix by the cancer cells

wherein a detection of cancer cell adherence, ingestion or invasion of the matrix is an indicator of cancer cells with metastatic potential.

6. A method for identifying an agent that inhibits the metastatic potential of cancer cells comprising:

(a) contacting the cancer cells and either a test agent or a vehicle control with a matrix coated with adhesion molecules;

(b) incubating the matrix for a time sufficient for adhesion and ingestion of the matrix by the cancer cells to occur; and

(c) detecting the adhesion and ingestion of the matrix by the cancer cells,

wherein a decrease in the adhesion and ingestion of the matrix by the cancer cells in the presence of the test agent, as compared to the adhesion and ingestion of the matrix by the cancer cells in the presence of a vehicle control, identifies a compound that inhibits metastases formation.

7. The method of claims 5 or 6 wherein the matrix is labeled with a fluorescent tag.

8. The method of claims 5 or 6 wherein the matrix is labeled with a radioactive isotope.

9. A composition for inhibiting the metastatic activity of cancer cells identified using the method of claim 6.

10. A method for inhibiting the metastatic activity of cancer cells in a subject comprising the administration of a seprase inhibitor.

11. A method for inhibiting the metastatic activity of cancer cells in a subject comprising the administration of a dipeptidyl peptidase IV inhibitor.

12. A method for identifying a nucleic acid that inhibits the metastatic potential of cancer cells comprising:

(a) introduction of the nucleic acid molecule into a cancer cell sample;

(b) inoculating said cancer cell sample onto a matrix coated with an adhesion molecule;

(c) incubating the matrix and cancer cell sample for a time sufficient for adhesion and ingestion of the matrix by the cancer cells to occur; and

(d) detecting the adhesion and ingestion of the matrix by the cancer cells,

wherein a decrease in the adhesion and ingestion of the matrix by the cancer cells containing the nucleic acid molecule, as compared to cancer cells not containing the nucleic acid molecule , identifies a nucleic acid molecule capable of inhibiting the metastatic potential of cancer cells .

13. The method of claim 12 wherein the nucleic acid molecule is an anti-sense molecule.

14. The method of claim 12 wherein the anti-sense molecule is a seprase antisense nucleic acid.

15. The method of claim 12 wherein the anti-sense molecule is a dipeptidyl peptidase IV anti-sense nucleic acid.

16. A method for removal of cancer cells from a sample derived from a cancer subject comprising contacting said sample with a matrix coated with an adhesion molecule for a sufficient amount of time to permit the adherence of cancer cells to the cell-adhesion matrix.

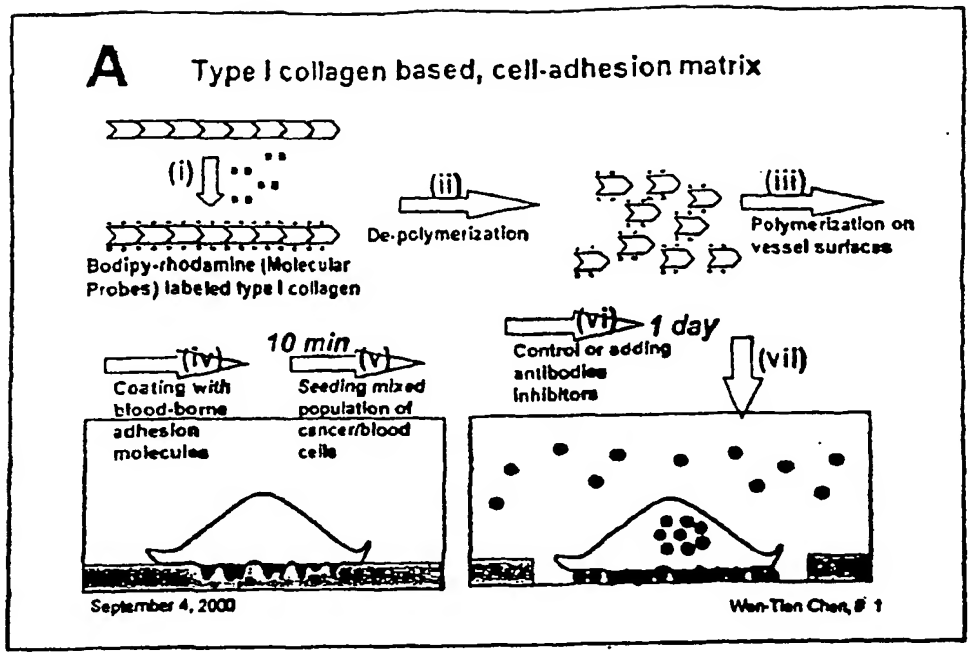
17. The method of claim 16 wherein said sample is an ascites, lymph, tumor or urine tissue sample.

18. A method for removal of cancer cells from the blood of a subject comprising contacting said blood with a matrix coated with an adhesion molecule for a sufficient amount of time to permit the adherence of cancer cells to the matrix.

19. The method of claim 18 wherein the blood sample from which the cancer cells have been removed is reintroduced into the subject.

20. A method for removal of cancer cells from the bone marrow of a subject comprising contacting said bone marrow with a cell-adhesion matrix coated with an adhesion molecule for a sufficient amount of time to permit the adherence of cancer cells to the cell-adhesion matrix.

21. The method of claim 20 wherein the bone marrow from which the cancer cells have been removed is reintroduced into the subject.


A
Type I collagen based, cell-adhesion matrix
(i)
(ii)
De-polymerization
(iii)
Polymerization on vessel surfaces
Bodipy-rhodamine (Molecular Probes) labeled type I collagen
10 min
(iv)
Coating with blood-borne adhesion molecules
(v)
Seeding mixed population of cancer/blood cells
(vi)
1 day
Control or adding antibodies inhibitors
(vii)
September 4, 2000
Wen-Tien Chen, # 1

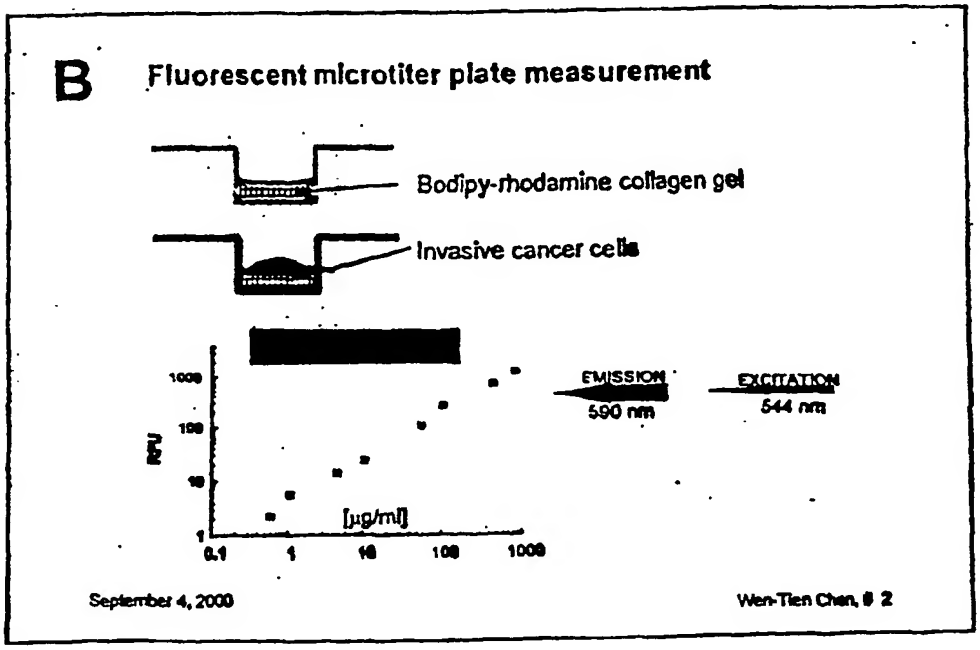
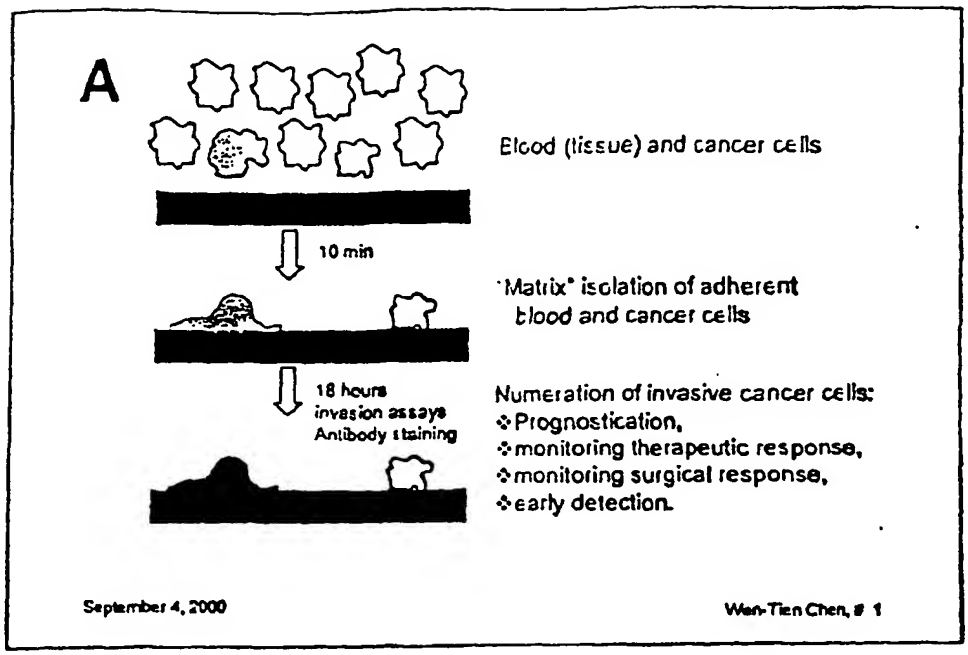

B
Fluorescent microtiter plate measurement
Bodipy-rhodamine collagen gel
Invasive cancer cells
EMISSION
590 nm
EXCITATION
544 nm
RFU
[µg/ml]
0.1
1
10
100
1000
September 4, 2000
Wen-Tien Chen, # 2

Figure 1A-B


A
Blood (tissue) and cancer cells
10 min
"Matrix" isolation of adherent blood and cancer cells
18 hours
invasion assays
Antibody staining
Numeration of invasive cancer cells:
Prognostication,
monitoring therapeutic response,
monitoring surgical response,
early detection.
September 4, 2000
Wen-Tien Chen, # 1

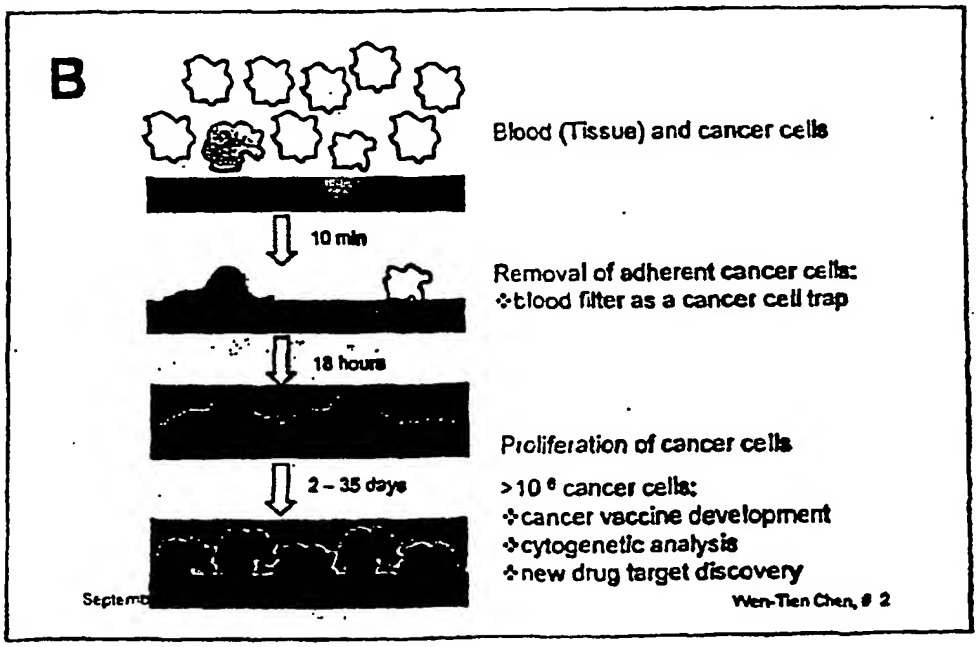
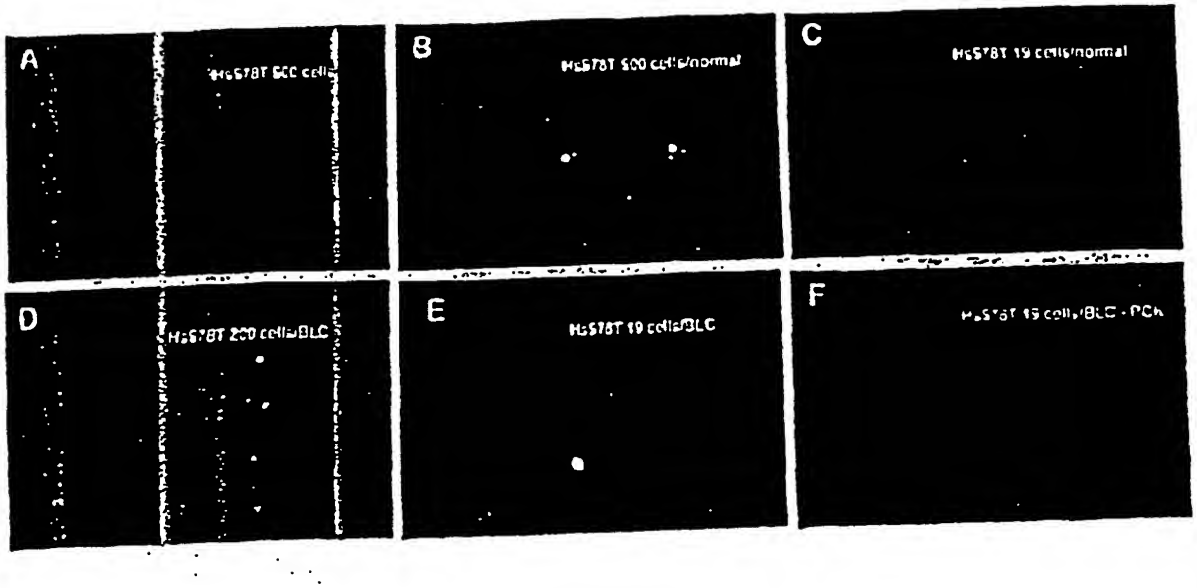

B
Blood (Tissue) and cancer cells
10 min
Removal of adherent cancer cells:
blood filter as a cancer cell trap
18 hours
Proliferation of cancer cells
2 – 35 days
>10 6 cancer cells:
cancer vaccine development
cytogenetic analysis
new drug target discovery
Wen-Tien Chen, # 2

Figure 2A-B

Figure 3



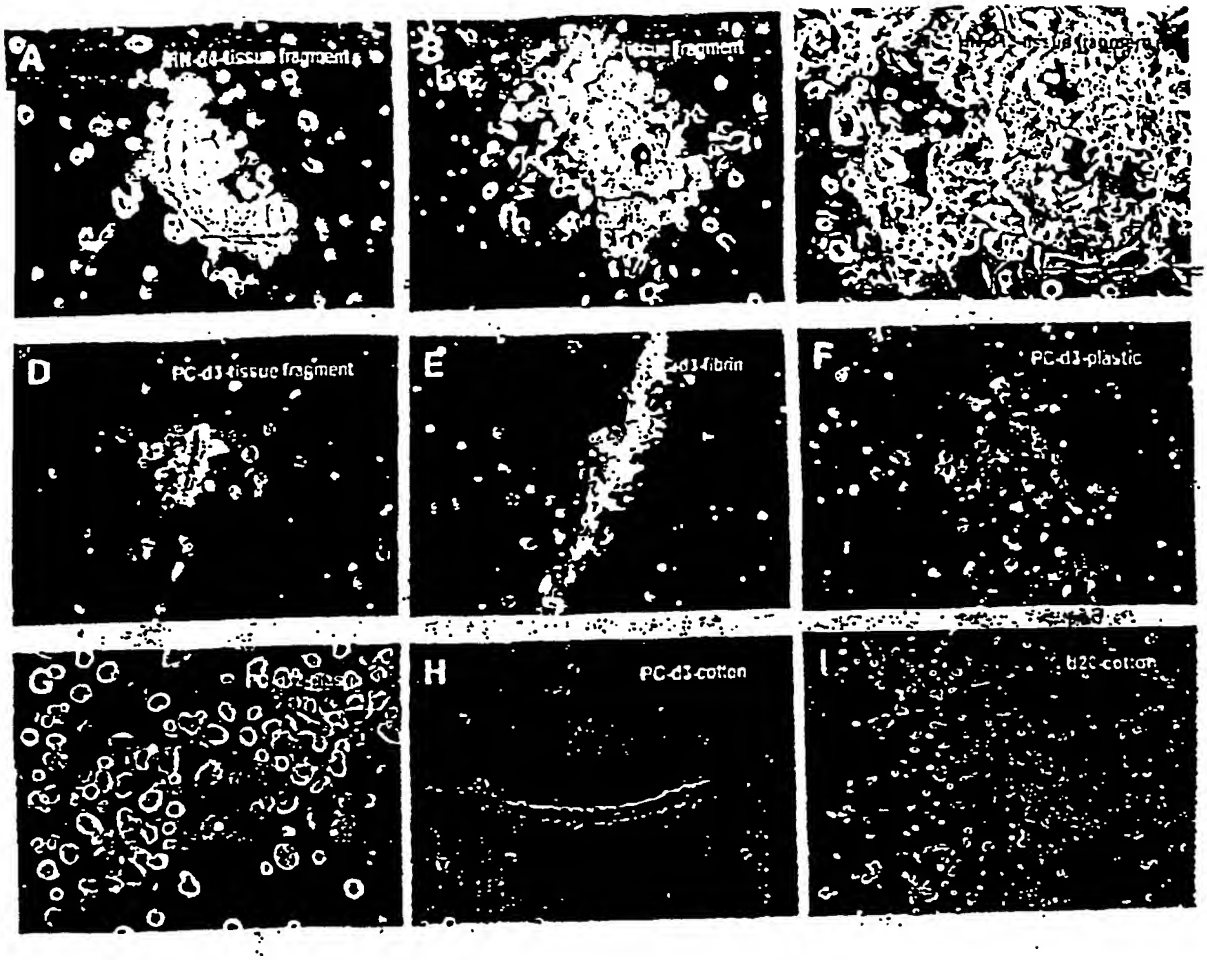
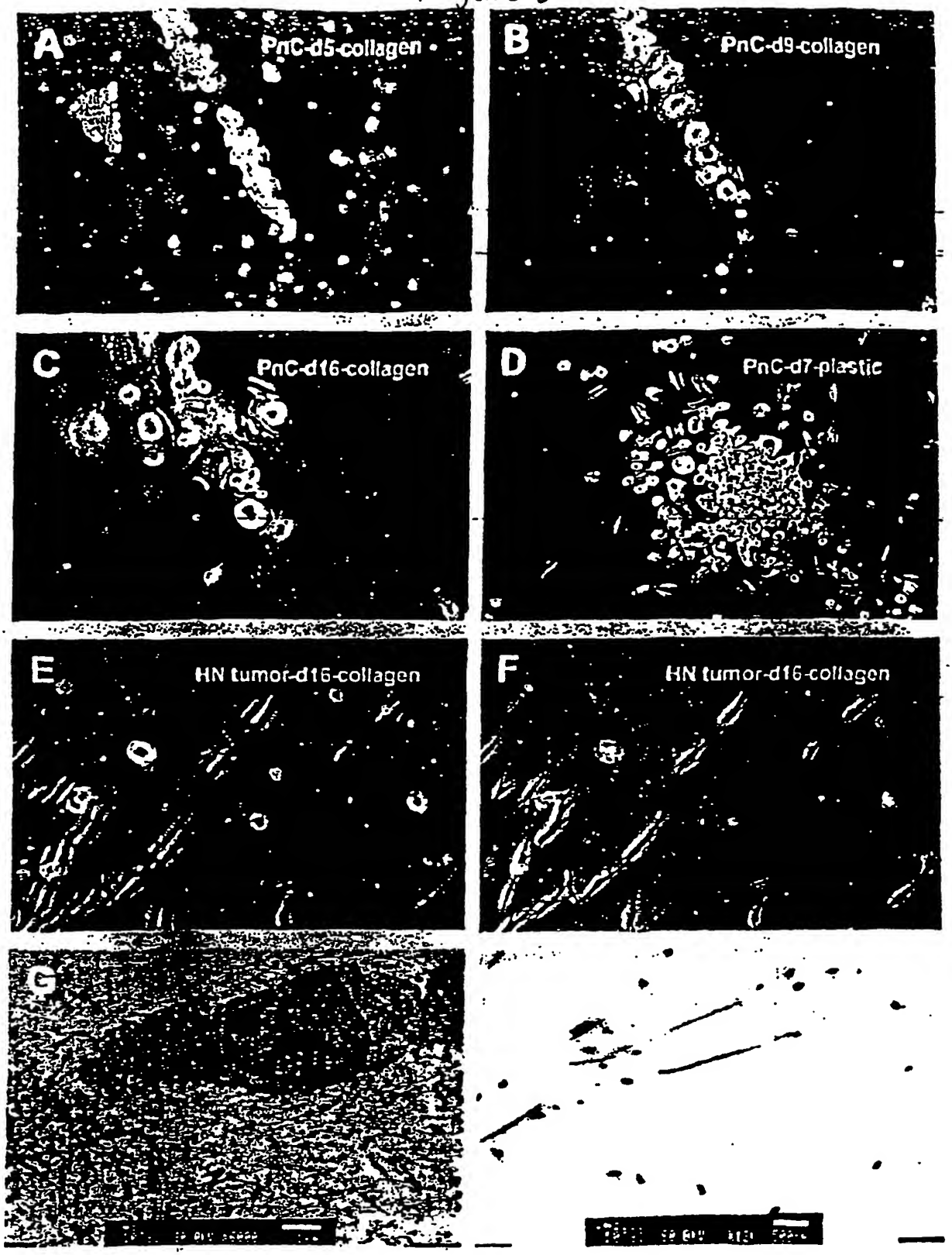

A
PC-d3-tissue fragment
B
tissue fragment
D
PC-d3-tissue fragment
E
F
PC-d3-plastic
H
PC-d3-cotton
I

Figure 4

Figure 5


A
PnC-d5-collagen
B
PnC-d9-collagen
C
PnC-d16-collagen
D
PnC-d7-plastic
E
HN tumor-d16-collagen
F
HN tumor-d16-collagen
G

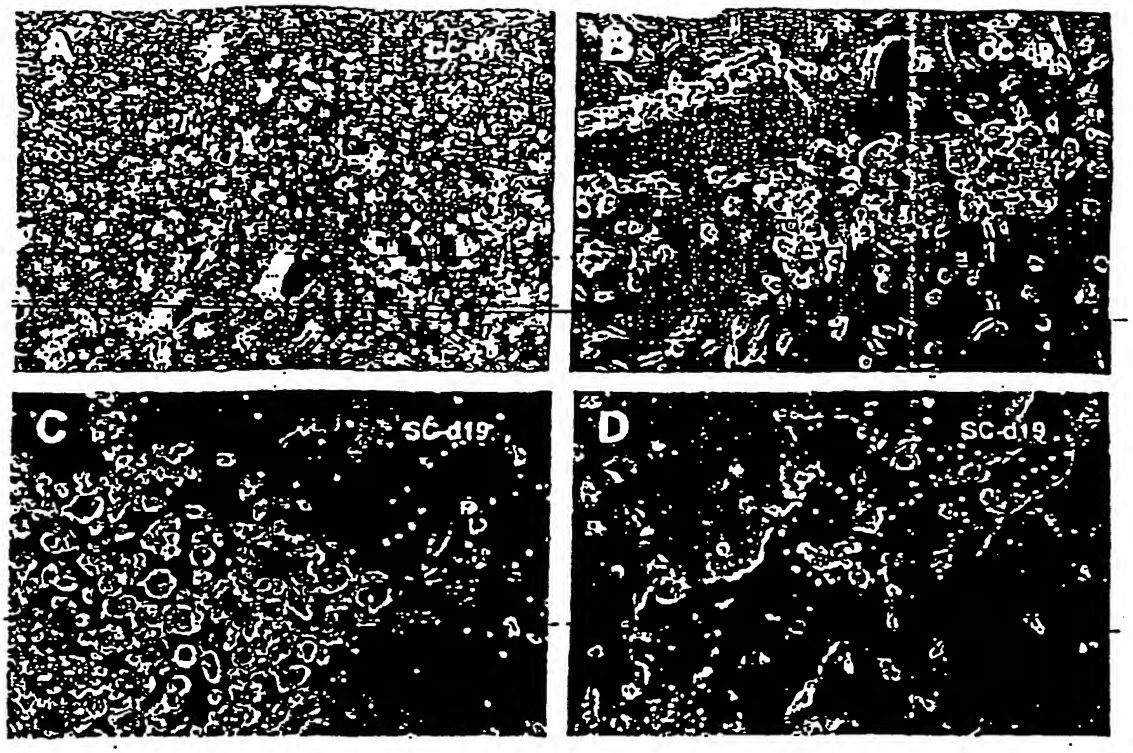


Figure 6

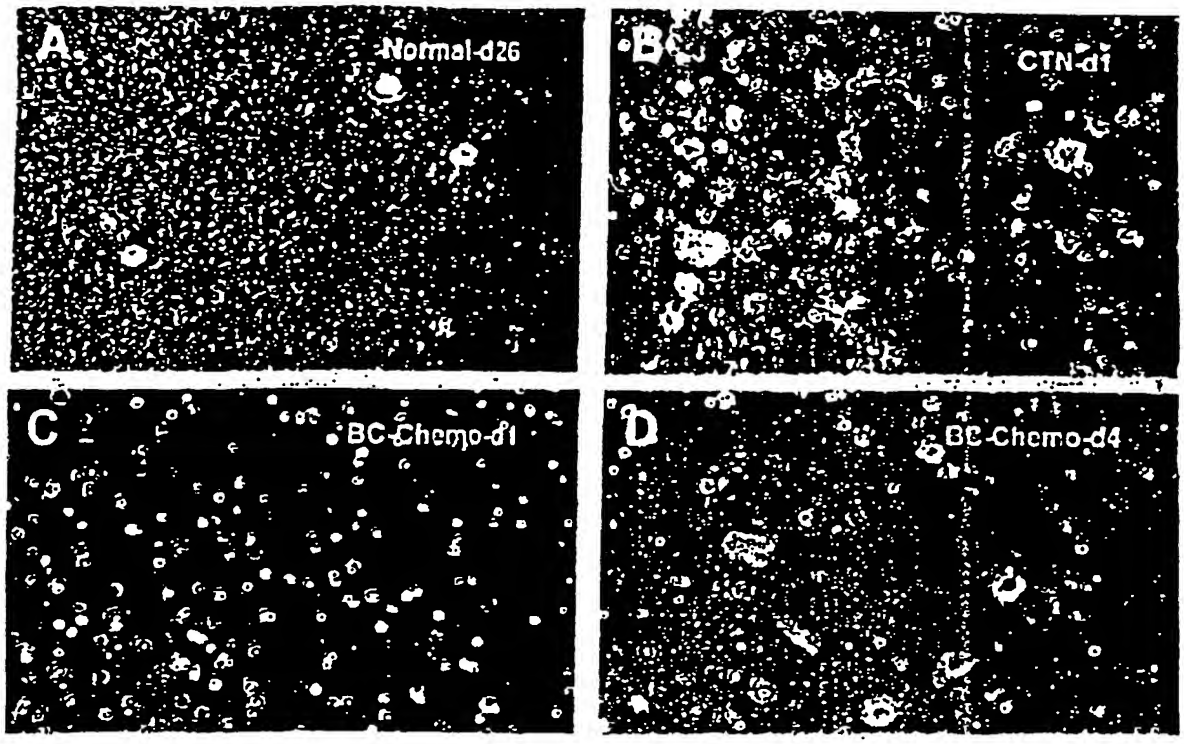


Figure 7

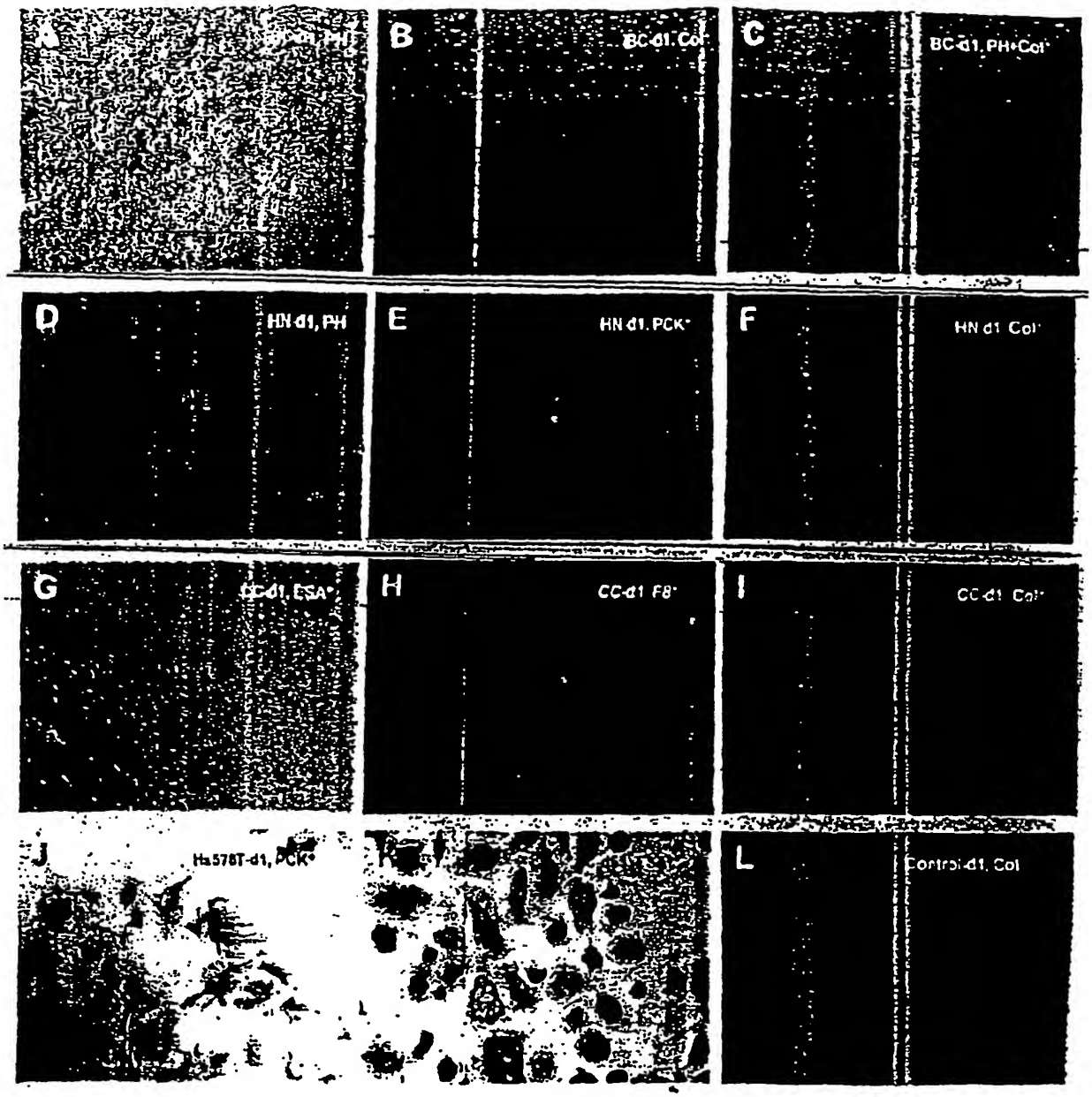

A
B
BC-d1, Col+
C
BC-d1, PH+Col+
D
HN-d1, PH
E
HN-d1, PCK+
F
HN-d1, Col+
G
CC-d1, ESA+
H
CC-d1, F8+
I
CC-d1, Col+
J
Ha578T-d1, PCK+
K
L
Control-d1, Col

Figure 8

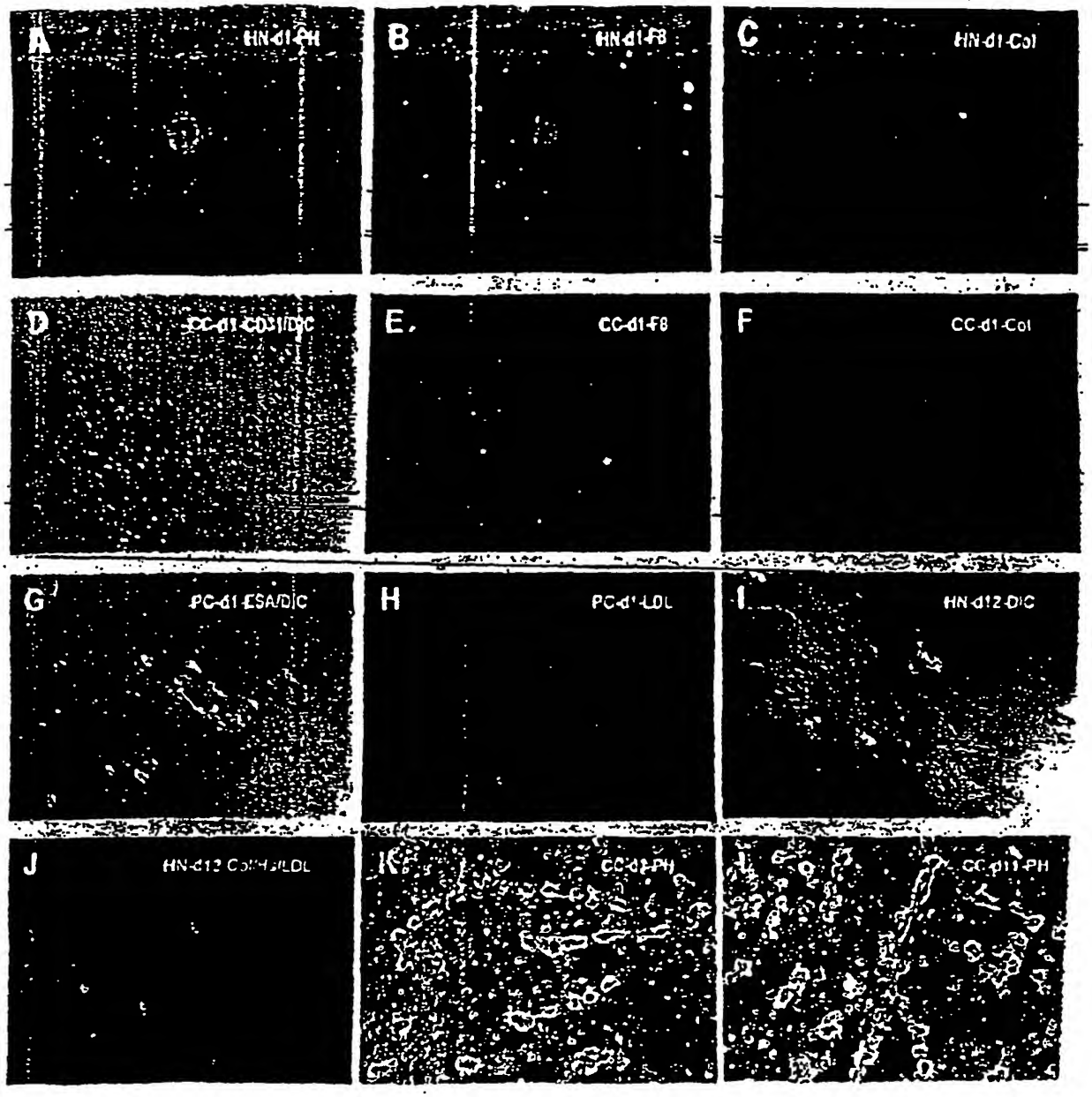

A
HN-d1-PH
B
HN-d1-F8
C
HN-d1-Col
D
CC-d1-CD31/DIC
E
CC-d1-F8
F
CC-d1-Col
G
PC-d1-ESA/DIC
H
PC-d1-LDL
I
HN-d12-DIC
J
HN-d12-CoH-a/LDL
K
CC-d1-PH
L
CC-d11-PH

Figure 9

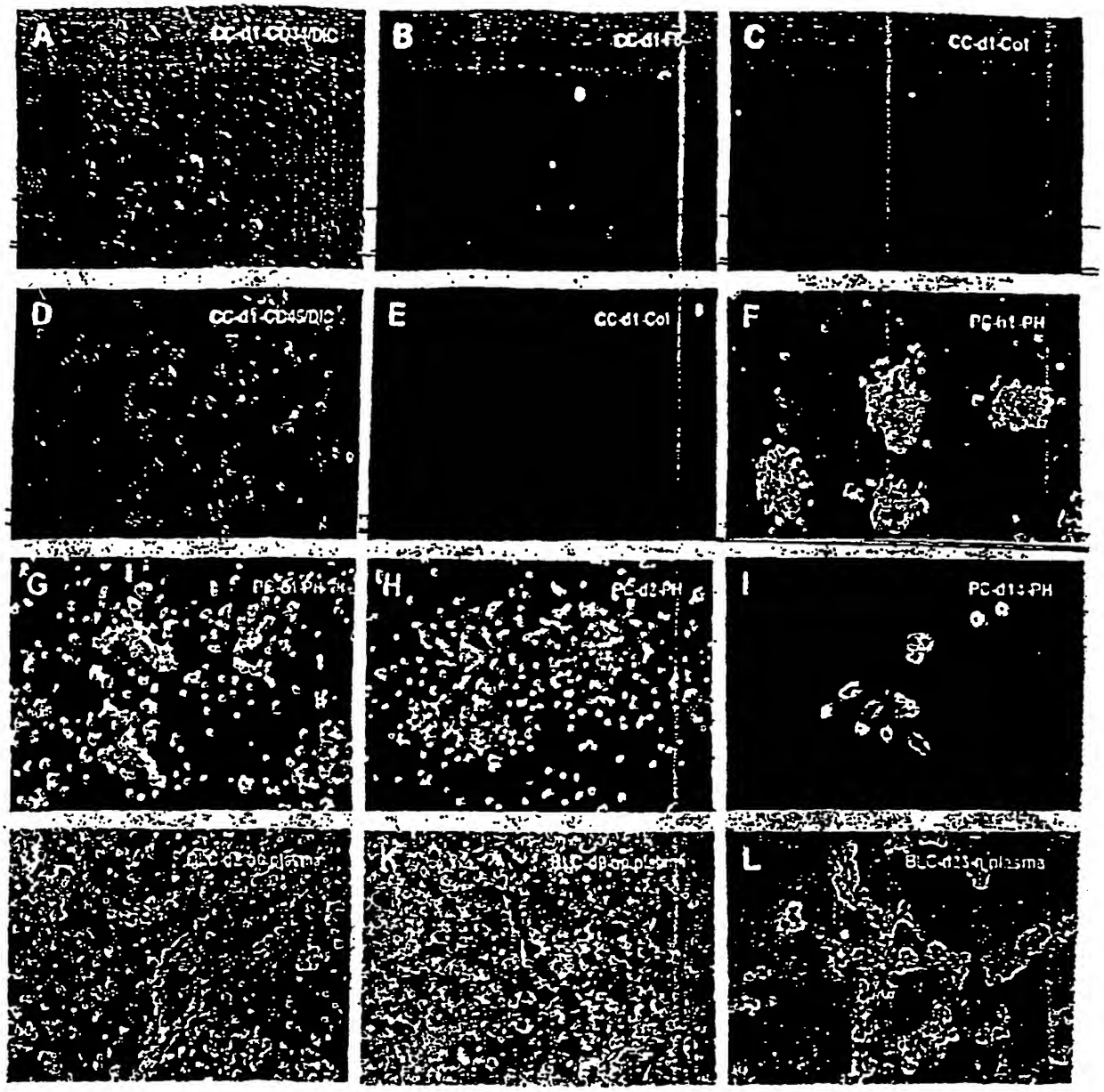


Figure 10

Figure 11

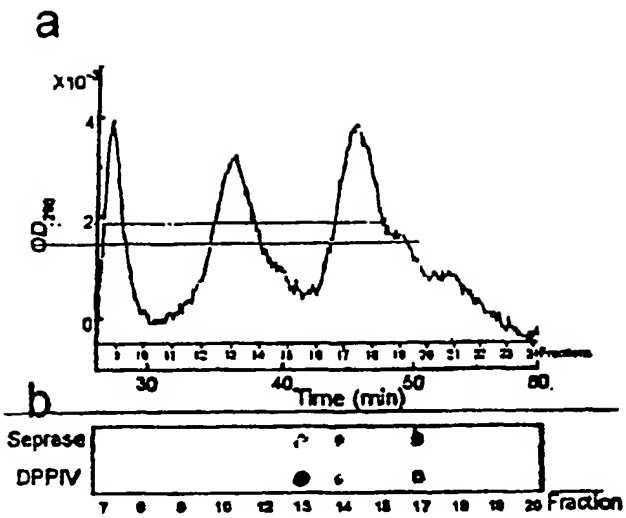


Figure 12

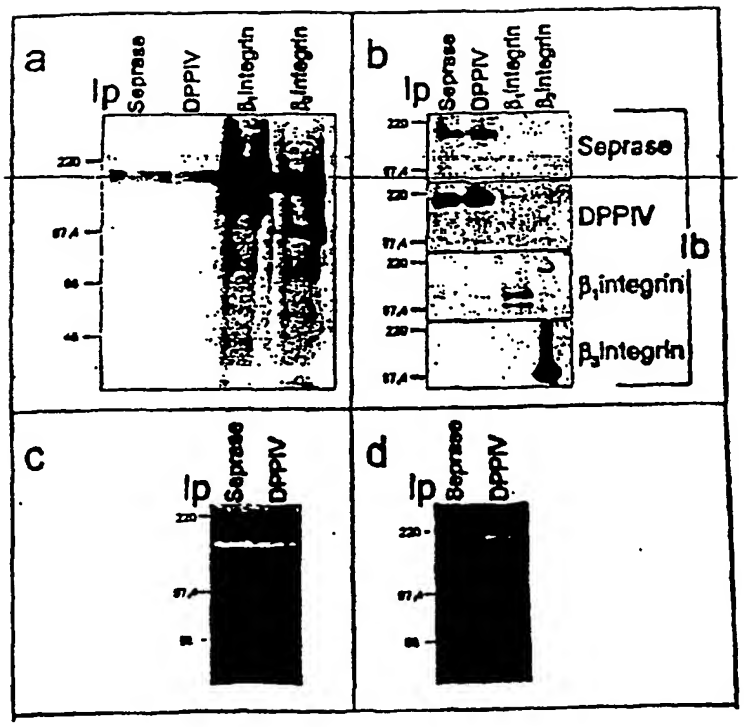

a
Ip
Seprase
DPPIV
β1integrin
β3integrin
220
97.4
66
46
b
Ip
Seprase
DPPIV
β1integrin
β3integrin
Seprase
DPPIV
β1integrin
β3integrin
Ib
c
Ip
Seprase
DPPIV
d
Ip
Seprase
DPPIV

Figure 13

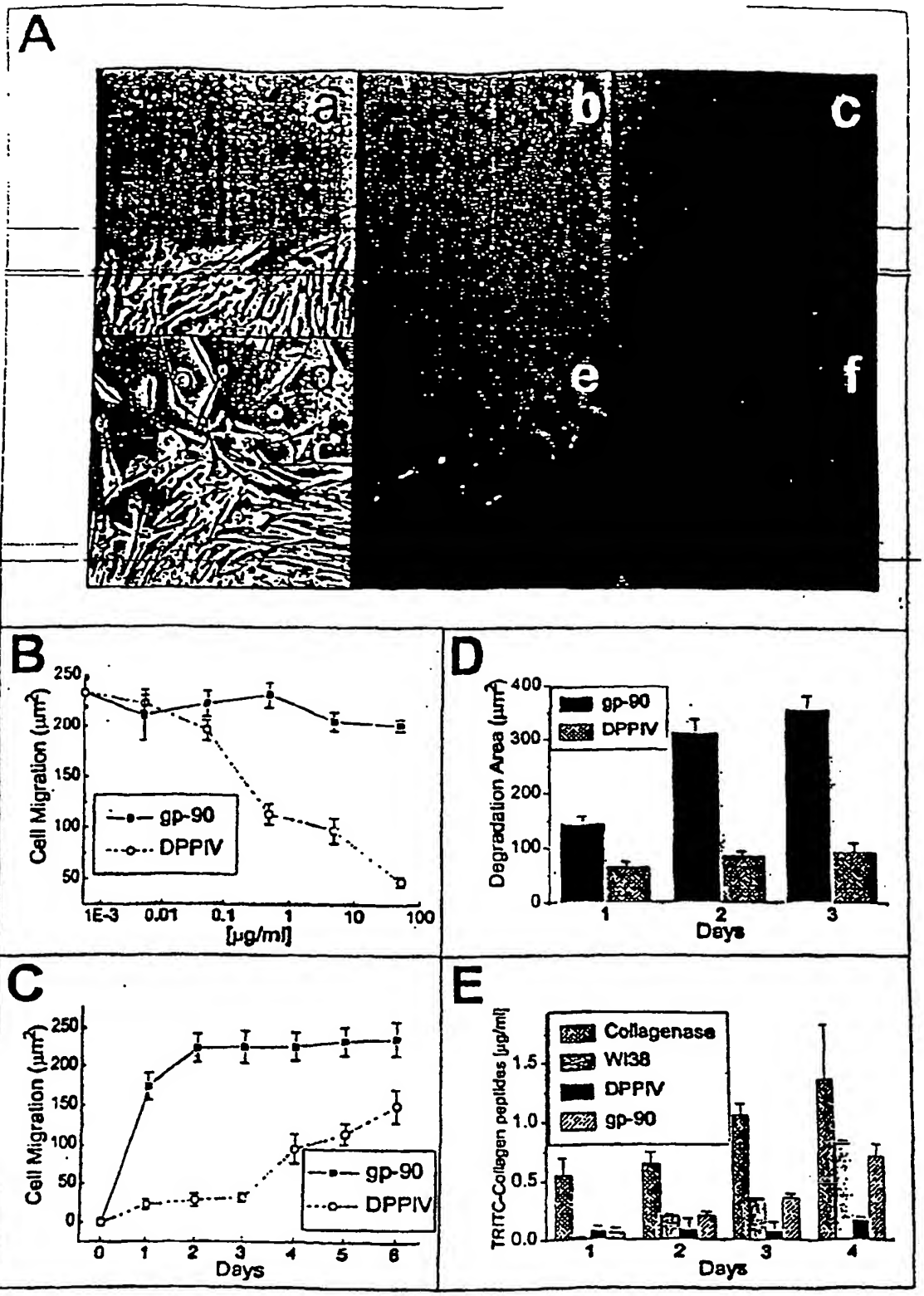

A
a
b
c
e
f
B
Cell Migration (μm²)
250
200
150
100
50
gp-90
DPPIV
1E-3
0.01
0.1
1
10
100
[μg/ml]
D
Degradation Area (μm²)
400
300
200
100
0
gp-90
DPPIV
1
2
3
Days
C
Cell Migration (μm²)
250
200
150
100
50
0
gp-90
DPPIV
0
1
2
3
4
5
6
Days
E
TRITC-Collagen peptides [μg/ml]
1.5
1.0
0.5
0.0
Collagenase
WI38
DPPIV
gp-90
1
2
3
4
Days

Figure 14

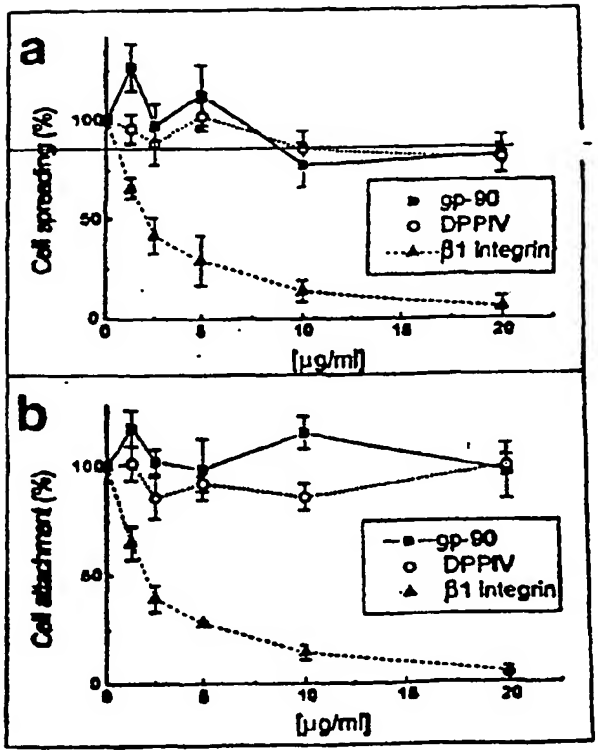


Figure 15

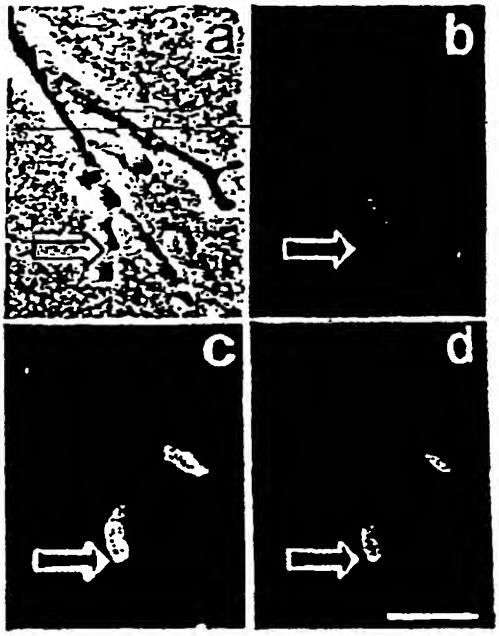


Figure 16

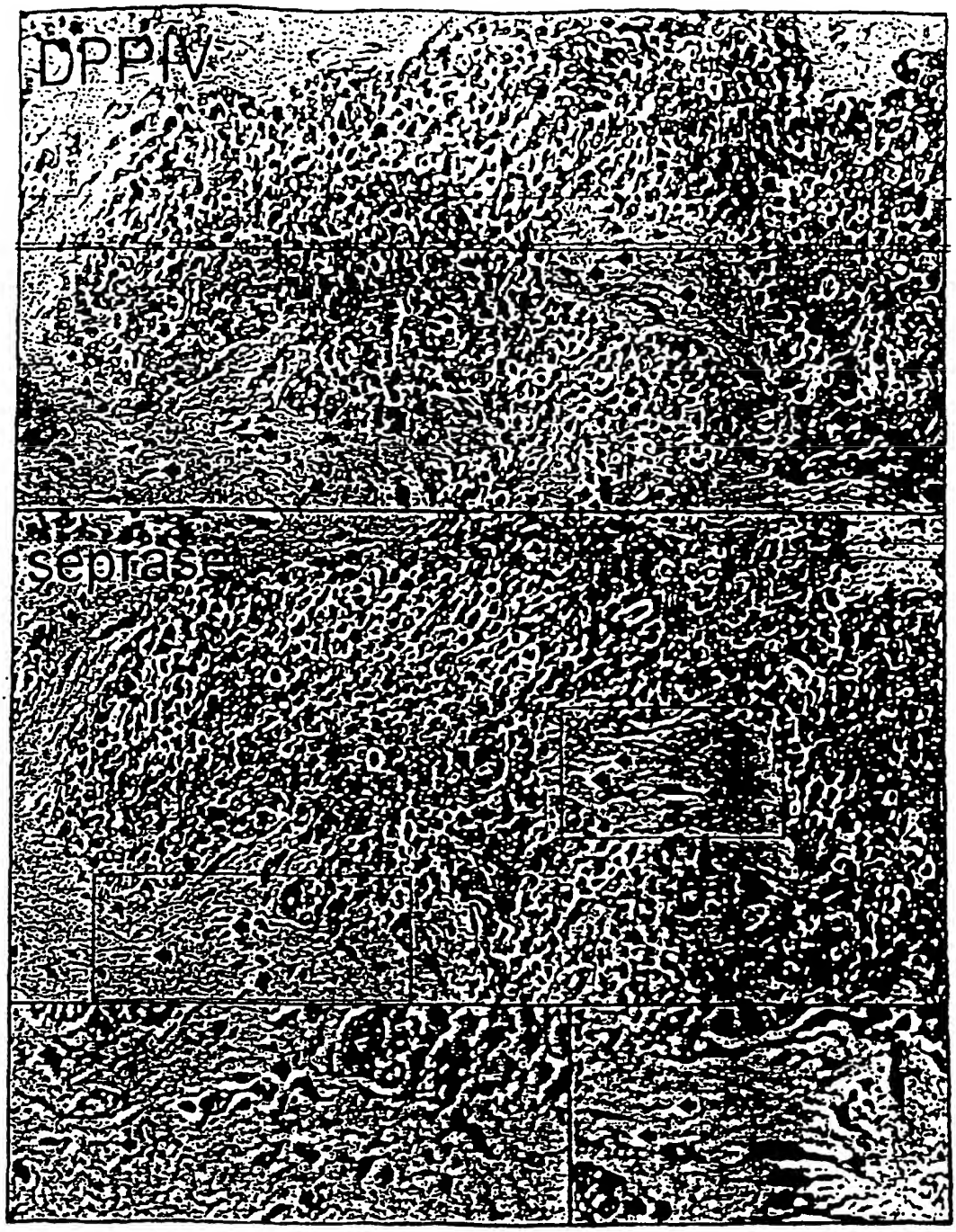
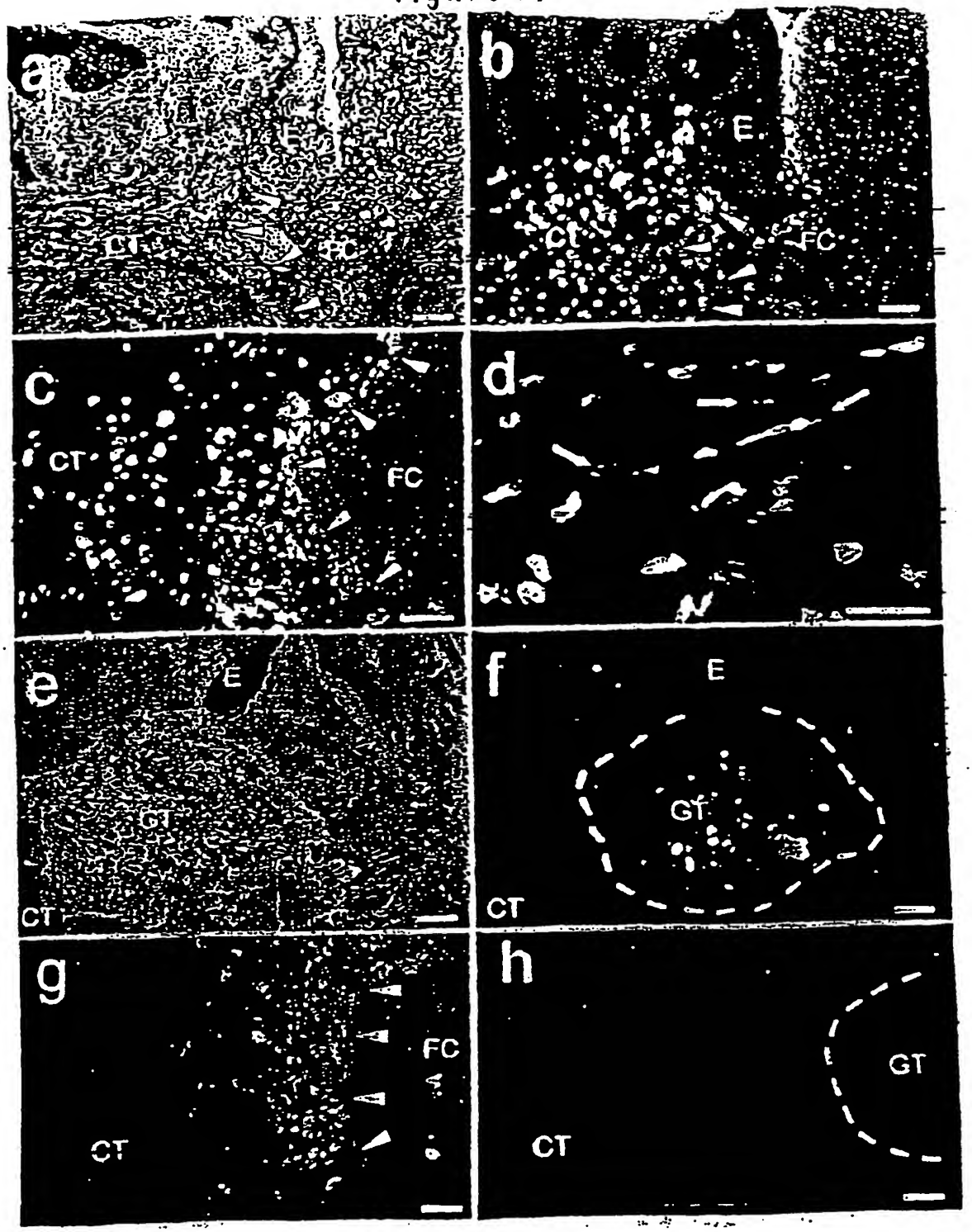


Figure 17



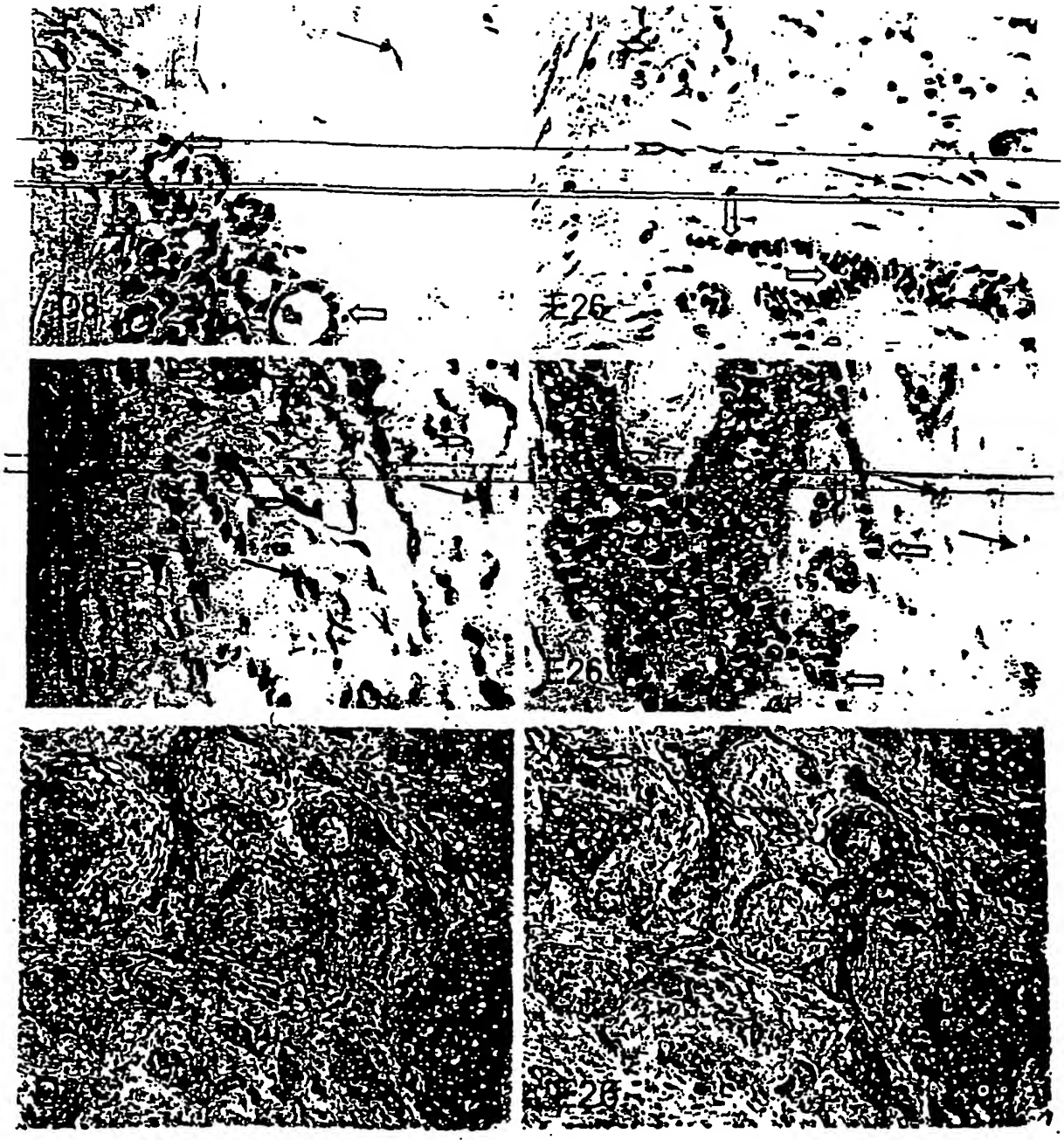


Figure 18

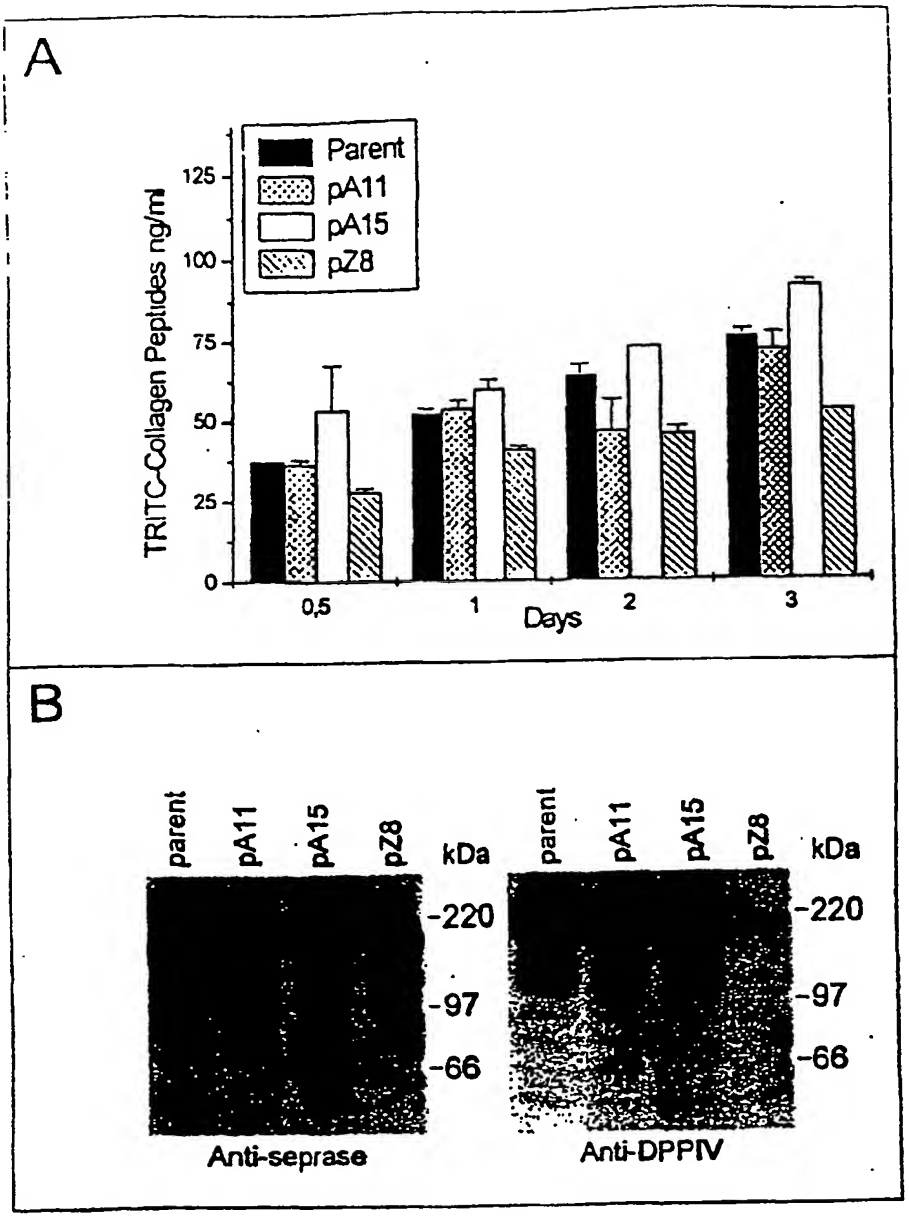

A
Parent
pA11
pA15
pZ8
TRITC-Collagen Peptides ng/ml
125
100
75
50
25
0
0,5
1
2
3
Days
B
parent
pA11
pA15
pZ8
kDa
-220
-97
-66
Anti-seprase
parent
pA11
pA15
pZ8
kDa
-220
-97
-66
Anti-DPPIV

Figure 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/26735

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/00; G01N 33/48

US CL : 435/4, 40.5, 40.51, 40.52; 436/63, 64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 40.5, 40.51, 40.52; 436/63, 64

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, CAPLUS, BIOTECHNO, JICST-EPLUS, BIOSIS, WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ALBINI, A. ET AL. A rapid in vitro assay for quantitating the invasive potential of tumors. Cancer Research. 15 June 1987, Vol 47, pages 3239-3245, see entire document	1, 4 and 5
Y	CANNISTRA, S. ET AL. Expression and function of beta one and alpha v beta 3 integrins in ovarian cancer. Gynecologic Oncology. 1995, Vol. 58, pages 216-225, see entire document.	1 and 3-5

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 26 NOVEMBER 2001	Date of mailing of the international search report 27 DEC 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Dorothea Lawrence for ALANA M. HARRIS, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998) ★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/26735

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-5

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/26735

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-5, drawn to a method for detecting the presence of cancer cells, wherein a detection of cancer cell adherence, ingestion or invasion of the cell-adhesion matrix is an indicator of cancer cells with metastatic potential.

Group II, claim(s) 6-8, drawn to a method for identifying an agent that inhibits the metastatic potential of cancer cells, hence decreasing the adhesion and ingestion of the matrix by the cancer cells in the presence of the test agent.

Group III, claim(s) 9, drawn to a composition for inhibiting the metastatic activity of cancer cells.

Group IV, claim(s)10 and 11, drawn to a method for inhibiting the metastatic acitivity of cancer comprising the administration of an inhibitor.

Group V, claim(s) 12-15, drawn to a method for identifying a nucleic acid molecule that inhibits the metastatic potential of cancer cells.

Group VI, claims 16-21, drawn to a method for removal of cancer cells.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I (claims 1-5), involving a method for detection is not required for Groups II-VI and there is no product associated with the instant group, hence there is no corresponding feature.

The special technical feature of Group III (claim 9), a composition is not required for Groups I, V and VI. Although this composition could possibly be used in Groups II and IV, this product is not listed as Group I or the first recited invention and accordingly is not linked to any other groups.

The special technical feature of Group V (claims 12-15), an anti-sense molecule is not required in the method groups of I, II, IV, nor VI.

The special technical feature of Group VI (claims 16-21), which features removal of cancer cells is not linked to Groups I-V.